PROGNOSTIC MARKERS IN OROPHARYNGEAL CANCERS

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List of abbreviations

AJCC- American joint committee on cancer
APC - antigen presenting cells
APC- annual percentage change
ASR- age standardised rate
BMI- body mass index
CD - cluster of differentiation
CI- confidence interval
CT- computed axial tomography
CTLA4 - cytotoxic T-lymphocyte-associated protein 4
DC- dendritic cells
DNA- deoxyribonucleic acid
EGFR- epidermal growth factor
ENT- ear, nose and throat
FACS - fluorescence activated cell sorter
GLUT - glucose transporter
HIF - hypoxia inducible factor
HNSCC- head and neck squamous cell carcinoma
HPV- human papilloma virus
HR- hazard ratio
IARC- international agency for cancer research
ICD- international classification of disease
IFN - interferon
IHC- immuno-histochemistry
ISH- in-situ hybridization
LRC- loco-regional control
MASP - mannose binding protein associated serine protease
MBP - mannose binding proteins
MHC - major histo-compatibility
MMP - metalloproteinases
mRNA- messenger ribonucleic acid
MSI - Multispectral imaging
NCCN- national comprehensive cancer network
NCT- national clinical trail
NK - natural killer cells
NLR - nod like receptors
OPSCC- oropharyngeal squamous cell carcinoma
OS- overall survival
PAMP - pathogen associated molecular patterns
PARSPORT- parotid sparing intensity modulated therapy versus conventional radiotherapy in head and neck cancer
PBM- pharygobasilar membrane
PD -1 - programmed death receptor 1
PD-L1 - programmed death receptor ligand 1
PET- positron emission tomography
RNS - reactive nitrogen species
ROS - reactive oxygen species
RTOG- radiation therapy oncology group
SCC- squamous cell carcinoma
SEER- surveillance epidemiology and end results
SIGN- Scottish intercollegiate guidelines network
TCR - T cell receptor
TIL – tumour infiltrating lymphocytes
TIM - T-cell immunoglobulin mucin
TLM- trans-oral laser microsurgery
TLR - toll like receptor
TNF - tumor necrosis factor
TOBTC- tonsils and base of the tongue
TORS- trans-oral robotic surgery
TPF- taxanes, platinum compounds and fluorouracil
UADT- upper aero-digestive tract
VHL - Von Hippel–Lindau
WHO- world health organisation
Kenneth Kenechukwu Oguejiofor, Doctor of Philosophy (PhD), The University of Manchester. Prognostic factors in Oropharyngeal cancers. March 2016

Introduction: Human papillomavirus (HPV) is changing the prevalence, survival and treatment paradigms in oropharyngeal squamous cell carcinoma (OPSCC). Improved survival of patients with HPV positive compared to HPV negative OPSCC has led to trials of treatment de-escalation. Current HPV detection methods are imprecise, therefore standardised assessment of transcriptionally active HPV in OPSCC is required. Furthermore, the differences in immune characteristics and/or the hypoxia response/effects could explain observed differences in prognosis between HPV positive and negative OPSCC. Rigorous HPV detection and subsequent biomarker evaluation should provide additional information required before introduction of treatment de-escalation in broad patient groupings.

Methods: The study cohort was 218 patients with OPSCC who received radiotherapy with curative intent. HPV status was determined on pre-treatment, formalin-fixed paraffin-embedded blocks using: 1) polymerase chain reaction (PCR); 2) in-situ hybridisation (ISH) and 3) immuno-histochemistry (IHC). QuantiGene multiplex assay was designed to detect mRNA of reference sequences of the common high-risk HPV types (16, 18, 33, 35, 45, 52 and 58). HPV detection methods were compared with mRNA quantification. Multimarker IHC of immune cell markers using chromogenic and fluorescent staining was performed, analysed and compared with single marker IHC using automated multispectral image analysis. A validated multiplex IHC method was used for a) chromogenic (CD3, CD4, CD8, and FoxP3) and b) fluorescent (CD8, CD68 and PD1/PD-L1) evaluation in tumour and stroma compartments. Single marker IHC was used to investigate tumour hypoxia markers (HIF-1α and CA-IX) in HPV positive and negative OPSCC.

Results: p16 IHC and ISH were the most sensitive and specific, respectively, for classifying HPV status. The combination of the three tests had the highest positive/negative predictive values compared with QuantiGene mRNA detection. Multiplex validation showed that, for serial sections up to 6 µm apart, there were highly significant correlations (P<0.0001) between single and multiplex counts for both chromogenic and fluorescent IHC. Overall there was less variation in cell counts with fluorescent staining when compared to chromogenic staining. Multiplex IHC of TILs in HPV positive and negative OPSCC showed higher infiltration in both tumour and stromal areas of CD3+CD4+ and CD3+CD8+ T cells but not CD4+FoxP3 Tregs in HPV positive compared with HPV negative OPSCC. Only CD3+CD8+ stromal and not tumour area infiltration was associated with increased survival (P=0.02). PD-L1 expression was higher in HPV negative OPSCC and this was related to macrophage (CD68) expression of PD-L1. In HPV negative tumours infiltration with CD68+PD-L1 was associated with a good prognosis. HPV negative patients had higher expression of HIF-1α but not CA-IX. High expression of both markers was associated with a poor prognosis irrespective of HPV status.

Conclusions: There are other prognostic factors operating in the larger subdivision of HPV positive and negative OPSCC. Precise HPV detection and inclusion of other prognostic factors is required before treatment de-escalation is used. Expression of immune inhibitory factors (PD1/PD-L1) alone without contextualisation with immune cell density is insufficient for patient prognostication and potential selection for therapy using immune checkpoint inhibitors. Hypoxia modification of radiotherapy should be explored in both HPV positive and negative OPSCC.
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**Authors biography**

I graduated in Medicine from the University of Jos in Nigeria in 2009. Whilst at medical school I developed an interest in oncology, this led me to obtain the Mres in Oncology at the University of Manchester and subsequently enrolled for a PhD. After this I will to continue my training as a junior doctor and apply for Clinical Oncology training positions. Also, I intend to maintain my links with translational cancer research whilst providing clinical care to patients.
1.0 Introduction

The principal function of translational research is to promote the translation of observations from laboratories into the clinic in a so called “bench-to-bed-side” process (Figure 1.1). For cancers this is particularly important given the constant evolution of information about oncogenesis and the time bound nature of diagnosis and treatment. Protocols for collaborations between clinicians and/or scientists are constantly being developed, evaluated and updated. Effective translational research will ultimately ensure that biomarkers identified/developed in the laboratory can properly be evaluated in patient groups. This novel information will potentially provide optimised “personalised medicine” accounting for inherent molecular heterogeneity of cancers both within and between different sites of origin or metastatic spread.

Genomic analysis shows that different types of cancers have distinct molecular origins (Cancer Genome Atlas 2012, Cancer Genome Atlas 2012), which presents both challenges and opportunities for therapy. Currently, genomic and or tissue based approaches can identify multiple oncogenic factors in a single patient sample that reflect both cellular and intercellular interactions. This potentially allows for a systematic analysis of hitherto known and/or unknown antigens/pathways. Multifactorial analysis (system biology) generates unique “signatures” reflecting specific conditions, cancers or individuals. This signature-based approach is a driving force for translational research methodology in oncology.

Oropharyngeal cancers (OPSCC), a subset of head and neck cancers, present an interesting challenge for translational research. Cancers originating from sites in the oropharynx are linked to differing aetiological origins and prognostic features. The aetiological and prognostic features are divided by positivity or negativity for the human papillomavirus (HPV). This dichotomy has important implications for observations of tumour biology and treatment stratification. It allows for the comparison of dissimilar cancers arising from the same anatomical site. Therefore, comparing biomarkers specific to each OPSCC sub-type and patient outcomes will increase understanding of the biology, prognosis and treatment selection of these cancer sub-type. The success of biomarker identification in the context of OPSCC will be measured by their impact on providing the most effective therapy for individual patients.
This thesis aims to provide further insights on OPSCC by identifying/characterising prognostic factors operational in the disease. Patients treated for OPSCC with a curative intent at a single tertiary site in a North-Western region of the United Kingdom were identified. Classification of aetiological origins, i.e. HPV positive or negative, was done using a combination of tests. All patients underwent radiotherapy. As radiation is known to augment the immune micro-environment, local immune response and factors related to the effectiveness of radiotherapy were also studied.

![Translational research workflow](image)

Figure 1.1 Translational research workflow

The evaluation of the role (if any) of the immune system in HPV positive versus negative OPSCC presents a technical challenge. Immune cell interactions with other cells (immune, invading microbes and/or host) are dynamic, and may vary in different parts of the tumour. Studies investigating these relationships should study the totality of such interactions. Analysis of individual markers in a single biopsy
may not be sufficient to provide this information. Multiplex analysis of several immune cell populations yields more information although ultimately is unlikely to detail completely immune activity in the whole tumour. A multiplex approach to the presence of key immune cell types is a good starting place to test for correlations between immune phenotypes and patient outcomes.

In OPSCC it is now evident that different HPV detection methods can produce different results. In Chapter 2 the development of a potentially clinically useful HPV mRNA detection is described and compared to the most popular detection method. Multiplex immunohistochemistry (IHC) methods are much improved as a result of availability of sensitive instrumentation and computer driven evaluation. However, multiplex IHC must be shown to be comparable to the current standard of single plex IHC before it is widely accepted for use. Chapter 3 presents a validation of a multiplex IHC method for both chromogenic and fluorescent methods. Subsequently, a multiplex IHC and automated image analysis method was used to evaluate the differential infiltration of immune cells into the compartments of the OPSCC microenvironment. Chapter 4 examines the immune cell markers CD3, CD4, CD8 and CD4FoxP3 in a chromogenic multiplex assay and relationships with the tumour microenvironment and outcomes. Chapter 5 examines CD8, CD68, CD8PD1, PD-L1 as a single and combination in a fluorescent multiplex.

Tumour hypoxia, a factor linked to limited cell killing following radiotherapy, is also evaluated in the context of HPV positive and negative OPSCC. Chapter 6 presents the data evaluating expression of hypoxia markers in HPV positive and negative OPSCC.

1.1 Head and neck cancers: oropharyngeal squamous cell cancers

Head and neck squamous cell carcinoma (HNSCC) are a heterogeneous group of malignancies arising from different anatomical sites of the upper aero-digestive tract (UADT) (Ragin et al. 2007). The majority of the squamous carcinoma originates from the oral cavity, oropharynx, hypopharynx and larynx (Ragin et al. 2007, Leemans et al. 2011). Cancers originating from the anatomical oropharynx are termed OPSCC. The overlapping anatomical boundary between the oral cavity and
the oropharynx presents a challenge for classification and classical epidemiology descriptions (de Camargo Cancela et al. 2010, Lambert et al. 2011).

### 1.1.1 Anatomy of the oropharynx

The oropharynx occupies the space posterior to the oral cavity; it begins at the lower borders of the mobile soft palate and extends inferiorly to the upper border of the epiglottis (Figure 1.2). The anterior wall is formed by the base of the tongue, which extends caudally to the vallecula of the epiglottis. Laterally the palatoglosus and palatopharygeous muscles form the anterior and posterior faucial pillars, respectively. These pillars enclose a triangular fossa that houses the palatine tonsils. The palatine tonsil, or “tonsil” as it is often referred, is covered by a capsule. There is lymphoid tissue at the base of the tongue. Thus the human oropharynx is lined by several immune structures. Histologically, the oral cavity and the oropharynx are lined with stratified squamous epithelium (Nave et al. 2001). This epithelium sits on dense collagenous fibrous tissue. However, in the tonsillar region the epithelium is slightly modified with numerous crypts extending from the under surface. These crypts are lined by stratified squamous epithelium interspersed with reticulated epithelium (Perry 1994, Nave et al. 2001). This reticulated epithelium is unusually thin and branched, thus presenting a larger surface area and a more intimate contact between the lymphatic cells and invading pathogens (Herrero 2003). Cancers can originate from any site in the oropharynx. The most common sites are the tonsils, base of the tongue, oropharyngeal wall and soft palate (Gillison et al. 2000, Ragin et al. 2007).

![Figure 1.2 Cross section of the anatomy of the head and neck showing the oropharynx.](image-url)
1.1.2 Epidemiology and risk factors of OPSCC

1.1.2.1 World

In 2012, the International Agency for Research on Cancer (IARC) reported that there were over 687,000 new cases and more than 376,000 deaths attributed to HNSCC worldwide. This makes HNSCC the sixth leading cause of cancer worldwide (Bray et al. 2015). IARC estimates that about 440,000 of these cancers originated in the mouth and the pharynx. About 65% of the cancers were in patients from the less developed parts of the world. There is a geographical variation with high incidence rates in south east Asia (Sri Lanka, India, Pakistan and Taiwan), parts of Europe (France, Hungary, Slovakia and Slovenia), parts of Latin America and the Caribbean (Brazil, Uruguay and Puerto Rico) and in the Pacific region (Papua New Guinea and Melanesia)(Warnakulasuriya 2009). The lowest incidence rates are reported in Africa, Central America and Eastern Asia. Men typically have higher incidence rates than women (Warnakulasuriya 2009, Lambert et al. 2011).

India has the highest incidence and prevalence; there were about 115,000 new cases of lip, oral cavity and pharyngeal cancers reported in 2012. In Pakistan, the estimated age standardised rates (ASR) (per 100,000) of oral and oropharyngeal cancers were 15.3 and 0.5 for men and 12.3 and 0.3 for women respectively (Ferlay et al. 2010). Similar high incidence rates are found in Sri Lanka where cancers of the oral cavity are the most common cancer in men (Warnakulasuriya 2009). In other parts of Asia cancers of the oral cavity and oropharynx are less common.

In the United States (US), IARC reported that there were around 33,000 cases of oral and pharyngeal cancers in 2008 (Ferlay et al. 2010). Similarly, the US Surveillance, Epidemiology and End Results (SEER) registries, reported that the age-standardised incidence rates of oral cavity and pharyngeal cancers between 2008 and 2012 was 16.5 per 100,000 for men and 6.3 per 100,000 for women (Kohler et al. 2015). In South America, the highest incidence rates are seen in Brazil (Warnakulasuriya 2009). Males in Brazil have the third highest rates worlwide (Wunsch-Filho and de Camargo 2001). This geographical variation in incidence is attributed to differences in exposure to the risk factors associated with these cancers (Table 1.1).

Similar trends are seen with mortality, about 75% of deaths from oral and pharyngeal cancers are in less developed regions (Ferlay et al. 2010). The highest
mortality rates are found in south central Asia (Lambert et al. 2011). Sankaranarayanan et al reported that the 5-year survival rates of tongue and oral cavity cancers in India were 23% and 37% respectively (Sankaranarayanan et al. 2010). In the US, SEER data reported mortality rates of 4.1 per 100,000 men and 1.5 per 100,000 in women between 2000 and 2004. Mortality rates were higher in African-American than in other racial groups (Warnakulasuriya 2009). African-Americans have 5-year survival rates of 39.5% which is lower than that for Caucasians (61.8%). Overall 5-year survival was about 59% (Ragin et al. 2007) (Table 1.2).

Table 1.1 Estimated age-standardised incidence rate from cancers of the mouth and pharynx for 100,000 individuals in some regions of the world in 2008.

<table>
<thead>
<tr>
<th>Region</th>
<th>Mouth</th>
<th>Oropharynx</th>
</tr>
</thead>
<tbody>
<tr>
<td>India (Mumbai)</td>
<td>6.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Men</td>
<td>6.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Women</td>
<td>3.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Pakistan (South Karachi)</td>
<td>15.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Men</td>
<td>15.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Women</td>
<td>12.3</td>
<td>0.3</td>
</tr>
<tr>
<td>France (Manche)</td>
<td>7.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Men</td>
<td>7.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Women</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>USA (9 SEER registries)</td>
<td>2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Men (white)</td>
<td>2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Women (white)</td>
<td>1.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Adapted from Lambert et al (Lambert et al. 2011)

Table 1.2 Estimated age-standardised rate of mortality from cancer of the mouth and in the pharynx for 100,000 individuals in different regions of the world in 2008.

<table>
<thead>
<tr>
<th>Region</th>
<th>Mouth</th>
<th>Pharynx</th>
</tr>
</thead>
<tbody>
<tr>
<td>North America</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Men</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Women</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Central, Eastern Europe</td>
<td>4.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Men</td>
<td>4.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Women</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>South Central Asia</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Men</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Women</td>
<td>3.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Adapted from Lambert et al (Lambert et al. 2011)
1.1.2.2 United Kingdom (UK) and the rest of Europe
In the UK in 2012, 9,387 new cases of oral cavity and pharyngeal cancers were reported with the incidence rates higher in males than females (Bray et al. 2015). Reports suggest that incidence rates are highest in Scotland (Conway et al. 2006). For the rest of Europe, there were about 135,000 people with oral and pharyngeal cancers in continental Europe (Globocan 2012). Central and Eastern Europe had the highest prevalence with around 51,000 cases. High incidence rates have also been reported in eastern European countries like Hungary. The lowest incidence rates were in Greece and Cyprus (Warnakulasuriya 2009). The lifetime risk of developing oral and pharyngeal cancers in Europeans is estimated at 1.85% for men and 0.37% for women (Warnakulasuriya 2009).

1.1.2.3 Trends over time
Studies from cancer registries across the world suggest an increasing prevalence of OPSCC in young, non-smoking, non-drinking men. This parallels declining incidence in cancers associated with tobacco smoking (Gillison et al. 2000, Shiboski et al. 2005, Reddy et al. 2010, Chaturvedi et al. 2011, Ramqvist and Dalianis 2011, Tinhofer et al. 2015). This increase has mostly been in cancers of the tonsils and base of the tongue. In a recent systematic review of OPSCC trends in the US, Stein et al reported an increase in the prevalence of HPV positive OPSCC from 20.9% in the pre-1990 time period to 51.4% in 1990–1999 and 65.4% for 2000 – present (Stein et al. 2015).

In England, Reddy et al analysed the incidence trends of tonsil and base of the tongue cancers from 1985-2006 and reported similar increases in the incidence in those aged 40-69 years (Reddy et al. 2010). In the Netherlands, Melchers et al analysed 193 patients over 15 years using a three method HPV detection algorithm and observed, increasing prevalence from 13% (1997 – 2004) to 30% (2005 to 2012) (Melchers et al 2015). Similar increases were seen in Sweden and Spain (Hammarstedt et al. 2007). There appears to be geographical differences in the prevalence of HPV positive OPSCC. It is pertinent to note that the observation of increasing prevalence of HPV positive OPSCC is mostly seen in western countries (Hashibe et al 2013). The changing prevalence of HPV positive OPSCC maybe a real effect but could also be the result of differing tumour classification schemes, HPV detection method and changes in the prevalence of non-HPV tumours at the
same site. Time trend studies notwithstanding, HPV is clearly an important risk factor in a significant proportion of OPSCC.

1.1.2.4 Risk factors

1.1.2.4.1 Smoking and alcohol

Historically tobacco and alcohol have been considered the key risk factors for the majority of oral and pharyngeal cancers (Ragin et al. 2007). Thus, cigarette smoking and alcohol were initially implicated in the development of OPSCC (Wynder et al. 1957, Blot et al. 1988, Franco et al. 1989, Choi and Kahyo 1991, Kabat et al. 1994, Gillison 2006). Subsequently, involvement of other tobacco products: bidis (tobacco hand-rolled in terburni leaf), cigars (shredded tobacco wrapped in tobacco leaf or paper), chuttas, snuff (ground tobacco), pipes and betel quid (betel leaves mixed with tobacco, areca nuts, lime wood, etc.) were shown to be risk factors (Hashibe et al. 2002).

A dose effect of tobacco and alcohol has been described (Blot et al. 1988, Franco et al. 1989). Cessation of smoking is associated with sharply reduced risks of OPSCC, approaching those of never smokers after 10 years (Blot et al. 1988, Franco et al. 1989). In most of the western world exposure to tobacco comes from cigarettes. In parts of South East Asia (India, Pakistan and Sri lanka) bidis, pipes, bethel quid, and snuff are the predominant forms of tobacco consumed. The smoke from tobacco contains a heterogeneous mix of chemicals. These include the polycyclic aromatic hydrocarbons, N-nitrosamines, aromatic amines as well as formaldehyde, phenol compounds and a variety of free radicals produced during tobacco combustion. The vantage position of the upper aero-digestive tract and its anatomy promotes an intimate interaction between its epithelium and these carcinogens. This interaction is further potentiated when a diluent like alcohol is present.

The interaction between tobacco and alcohol is considered responsible for about 80% of oral and oropharyngeal cancers (Gillison 2007, Anantharaman et al. 2011). Presently it is unclear how alcohol causes cancers (Gillison 2007). However, several epidemiological studies have linked alcohol to the development of oral cancers (Wynder et al. 1957, Graham et al. 1977, Gronbaek et al. 1998, Kjaerheim et al. 1998, Boffetta et al. 2001). In never smokers, Hashibe et al describe similar dose effects of alcohol and head and neck cancers as seen with smoking (Hashibe et al 2007). Other work suggests that alcohol may mediate its effects through the
induction of p450 cytochrome enzymes (Roberts et al. 1995) or the inhibition of phase II enzymes involved in clearance of carcinogens (Singletary and Gapstur 2001). Similarly, available evidence suggests that acetaldehyde (a metabolite of alcohol) may disrupt cell cycle, apoptosis and DNA repair mechanisms (Seitz et al. 1998). A pooled analysis of 15 case-control studies (9,107 cases, 14,219 controls) reported comparative relative risks of developing HNSCC for beer and liquor but weaker associations with moderate wine consumption (Purdue et al. 2009).

Presently, smoking and alcohol effects account for about 74% of HNSCC worldwide (Anantharaman et al. 2011). This influence is dwindling in western countries as smoking cessation programmes are implemented (Sturgis and Cinciripini 2007, Dahlstrom et al. 2008, Kim et al. 2010). Similarly, in younger patients the population attributable fractions (PAF) for smoking and drinking are lower when compared with older adults (Toporcov et al. 2015). Studies have investigated the role of involuntary smoking. A pooled analysis reported that long duration of involuntary smoking exposure at home and at work increased risk of HNSCC. This effect was stronger for pharyngeal and laryngeal cancers than any other sub-site (Lee et al. 2008).

1.1.2.4.2 Familial/genetic predisposition, diet and body mass index
A familial and genetic predisposition to developing head and neck cancers have been reported (Goldgar et al. 1994, Goldstein et al. 1994, Garavello et al. 2008, Negri et al. 2009). In OPSCC this association was reported as weak (Goldstein et al. 1994) or strong (Garavello et al. 2008). In a genome wide association study, Mckay et al investigated genetic variants in 8,605 cases of HNSCC and 16,226 controls. They reported evidence of a significant association between HNSCC susceptibility and five variants: three previously identified (rs1573496-ADH7, rs1229984-ADH1B and rs698-ADH1C) and two novel variants 4q21 (rs1494961) and 12q24 (rs4767364) (McKay et al. 2011).

Similarly, others have reported an association between body mass index and oral and pharyngeal cancers (Kabat et al. 1994, D'Avanzo et al. 1996, Franceschi et al. 2001, Hashibe et al. 2002, Nieto et al. 2003, Rodriguez et al. 2004, Kreimer et al. 2006). These studies agree that a lean body mass could predispose or serve as an early marker (Franceschi et al. 2001). In a pooled analysis of 17 case-control studies of 12,716 cases and the 17,438 controls, Gaudet et al reported that the adjusted odd ratio (95% CIs) were elevated for people with BMI at reference (date of diagnosis for cases and date of selection for controls) ≤18.5 kg/m² and reduced for BMI >25.0–30.0 kg/m² and BMI ≥30 kg/m², compared with BMI >18.5–25.0 kg/m². These associations did not differ by age, sex, tumour site or control source. Although the increased risk among people with BMI ≤18.5 kg/m² was not modified by tobacco smoking or alcohol drinking, the inverse association for people with BMI>25 kg/m² was present only in smokers and drinkers (Gaudet et al. 2010). The interdependence of the lifestyle factors above confounds the singular contribution of particular lifestyle factors. Furthermore, the preventive/permissive influences of these factors when inundated by more established risk factors, i.e. smoking and/or HPV, are yet to be described.

1.1.2.4.3 Human papilloma viruses
Syrajenen first hypothesised a possible aetiological link between HPV and oral cancers when he observed morphological and immuno-histochemical evidence of HPV infections in oral cancer biopsies (Syrjanen et al. 1987). Several other investigators have reported on the presence of HPV DNA in different anatomical locations of the head and neck (Loning et al. 1985, Niedobitek et al. 1990, Gillison et al. 2000). Gillison et al reported a HPV prevalence rate of 6.9% in the normal epithelium of men and women aged 14 to 69 years with a peak prevalence at 30 to 34 years (Gillison et al. 2012). It is presumed that as in cervical cancer persistent high risk HPV infection will be a risk factor for development of OPSCC, but it is not known what percentage regress or progress to cancer. In oropharyngeal cancer, detection rates of HPV DNA quoted are for tonsil and tongue base sites (Gillison et al. 2012). HPV 16 is the most common type detected in 87.6% of HPV associated OPSCC (Kreimer et al. 2005).
It is clear that in some western populations, HPV is challenging other risk factors historically associated with oral and pharyngeal cancers, such as smoking and alcohol. It has been suggested that sexual transmission might be the means of HPV infection (Gillison et al. 2000, Gillison et al. 2008, Attner et al. 2011). D’souza et al reported that a high lifetime number of vaginal-sex partners ≥ 26 was associated with an increased risk of OPSCC, as was a high lifetime number of oral-sex partners (≥6) and that the degree of association increased with the number of vaginal-sex and oral-sex partners (P = 0.002 and 0.009, respectively) (Hemminki et al. 2000, D'Souza et al. 2007). Another study reported that patients with HPV associated ano-genital cancers have a 4.3-fold increased risk of tonsillar SCC (Frisch and Biggar 1999). In that study, other HNSCCs occurred but the relative risk of cancer development was significantly lower than for tonsillar SCC. Among patients with HPV unrelated cancers the relative risk was close to 1.0 (Frisch and Biggar 1999).

A pooled analysis of four populations and four hospital-based studies reported that cancer of the oropharynx was associated with a history of six or more lifetime sexual partners and four or more lifetime oral sex partners. Cancer of the tonsil was associated with four or more lifetime oral sex partners and, among men, with ever having oral sex and with an early age of first intercourse. Cancer of the base of the tongue was associated with ever having oral sex among women, having two sexual partners in comparison with only one and, among men, with a history of same-sex sexual contact (Heck et al. 2010). This seems to support the role of sexually transmitted HPV in the development of oral and pharyngeal cancers.

In HPV positive cervical cancers, transcripts of the viral genes E6 and E7 are detectable and can act as oncogenes when the expression is no longer regulated by viral gene E2 and/or differentiation of the cell (Nakagawa et al. 2000, Burd 2003). It is not known if the mechanism of oncogenesis is similar in HPV positive OPSCC and cervical cancers. Van Houten et al studied 84 HNSCC, 20 of the tumours were HPV positive with PCR. Of these, 10 tumours were consistently positive for HPV DNA using others assays. Nine out of 10 showed E6 mRNA activity. These nine E6 mRNA-positive cases all lacked a TP53 mutation, whereas both the other HPV DNA-positive and HPV-DNA negative tumours had TP53 mutations in 36% and 63%, respectively (van Houten et al. 2001), a finding corroborated by others (Wiest et al. 2002, Braakhuis et al. 2004). On the basis of this evidence high risk HPV is
now recognised as a risk factor for the development of oral and pharyngeal cancers by the IARC (Ramqvist and Dalianis 2011).

The increasing incidence of HPV positive OPSCC has been described as an “epidemic” by some investigators (Junor et al. 2010, Ramqvist and Dalianis 2011). It is obvious that HPV is changing the landscape of HNSCC. However, infection with high-risk HPV types does not always lead to the development of cancers. In the cervix, precancerous lesions do regress. Therefore, high risk HPV infection is considered necessary but insufficient for cancer per se. HPV types 16 and 18 account for about 40-60% of oropharyngeal cancers (Gillison 2004, D'Souza et al. 2007, Anaya-Saavedra et al. 2008, Fakhry et al. 2008), of which 80-90% are associated with HPV 16 (Herrero et al. 2003, Kreimer et al. 2005, Gillison 2007). In cervical cancers around 12 HPV types in alpha 7 and alpha 9 species are responsible all of the cancers with HPV 16, 18, 31, 33, and 45 being the most prevalent (Kreimer et al 2007).

1.2 Human papilloma viruses and cancer

1.2.1 Classification

The papillomaviruses belong to the papillomaviridae family. They infect epithelial tissues in mammals, amphibians, reptiles and birds (Chow et al. 2010). There are about 150 papillomavirus types belonging to several genera (named using the Greek letters alpha to pi), and each has several species (de Villiers et al. 2004). Papillomaviruses specific to humans (HPV) belong to five genera (Alpha-, Beta, Gamma-, Mu-, and Nu-). Historically papillomaviruses were initially classified as papovaviridae due to their similarities with the polyomaviruses, but it is now known that the similarities have no taxonomic significance (Bernard et al. 2010) and they are recognised as two separate families by the International Committee on the Taxonomy of Viruses (ICTV) (de Villiers et al. 2004). These viruses cause benign infections/tumours and/or malignant tumours in infected organisms (Figure 1.3).

The structural proteins are used to classify papillomavirus into the different types and or genus. Less than 60% homology is seen between papillomavirus types in the different genera. The L1 region on the papillomavirus is well conserved across papillomavirus types and its nucleotide sequence is used for this classifications. A
sequence difference of less than 2% is considered an intra-type variant (de Villiers et al. 2004, Bernard et al. 2010).

The alpha HPV types are pathogenic to human cutaneous and mucosal surfaces (Doorbar et al. 2012). These HPV types are divided into “high risk” and “low risk” types based on their ability to cause cancers (Bernard et al. 2010). Cutaneous alpha types are mostly low risk types (Doorbar et al. 2012) whereas mucosal types can be either low or high risk (Bosch et al. 2008). Low risk mucosal alpha types (alpha 10 [types 6 and 11]) are associated with genital warts and juvenile respiratory papillomatosis (Gerein et al. 2005, Hsueh 2009, Donne and Clarke 2010). High risk mucosal types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) are associated with carcinomas of mucosal sites in the ano-genital and oropharynx regions, and have been classified as carcinogens by the World Health Organisation (WHO) (Bouvard et al. 2009).

HPV infection patterns show either a bimodal or unimodal trend in different parts of the world (Matos et al. 2003, Hamlin-Douglas et al. 2008). HPV clearance (defined as two negative test following a positive test) has been reported in population based studies (Ingles et al. 2015). Factors responsible for successful clearance remain largely unknown. Reports suggest that 66% and 90% of infections are cleared in 12 and 24 months respectively (Whang et al. 2015). Persistence of HPV infection is linked to high viral load. However, others show no link with smoking, number of sexual partners, oral contraceptive use, and high parity (Miranda et al. 2013). As persistence of HPV is a prerequisite for oncogenesis it is pertinent that factors responsible for clearance and/or persistence in different regions are elucidated.

1.2.2 Structure and replication

1.2.2.1 Structure
Unravelling the structure and mechanism/life-cycle of HPV infection predicates the development of detection, prevention and therapeutic strategies against the virus. HPVs are non-enveloped double stranded DNA viruses with about 8,000 base pairs in their genomes. The HPV genome is a circular, double stranded DNA and forms chromatin like structures in association with histones (Conway and Meyers 2009). The genome has eight open reading frames (ORF) which encode for two “late” (L1 and L2) and six “early” (E1, E2, E4, E5, E6 and E7) genes (Doorbar et al. 2012).
The genome also contains a non-coding “upstream regulatory region” (URR) (Buck et al. 2008). The URR serves as the site for DNA replication, and promoter and transcription factor binding (Chow et al. 2010).

The early gene proteins are transcribed early in the viral life cycle (Conway and Meyers 2009), and this is preceded by the activity of the early promoter (Smith et al. 2007). The early promoter or P1 is located upstream of the E6 gene and controlled by the E2 rich binding site at the 3’ end of the URR (Chow et al. 2010). The early gene proteins when produced are involved in viral replication and proliferation in infected cells. The late genes that are transcribed late in the infection cycle encode for structural/capsid proteins. The viral life cycle is integrated with the differentiation of the epithelial tissue, which it targets.

Structurally the capsid proteins form from 72 pentameric capsomeres arranged in an icosahedral lattice (T=7) (Figure 2) (Lowe et al. 2008); there are about 10 times more L1 than L2 in the capsids. These capsomeres form a continuous shell 1.2 and 3.1 nm thick. Interspersed on the shell are <0.5nm gaps, and the outer surface of the capsid shell is more uneven than the inner surface. The L1 protein has a centrally located thick region with a diameter of about 8.6 nm, radiating from this are five heads (pentamers). The pentamers are only in contact with each other at the central region and are separated at the distal heads. The L1 protein is primarily responsible for the structure of the capsid, its helices interact via intra and inter pentameric interactions to stabilize the overall structure (Zhou et al. 1994). The L1 protein can aggregate into pentavalent or hexavalent structures (T=1). The final T=7 structure does not form readily in vitro. Virus like particles (VLP) can be assembled by modifying the amino acids at the C-terminus and N-terminus of the L1 proteins. These VLPs aggregate into 12 L1 pentamers with a T=1 structure. High levels of type specific neutralizing antibodies are generated after immunisation with this HPV L1 VLP (Schiller and Hidesheim 2000).

1.1.2.2 Replication
The HPV life cycle begins with infection of basal keratinocytes (Stanley 2008, Doorbar et al. 2012), which is mediated by the interaction of the virus particle with heparan sulphate proteoglycans present on the surfaces of epithelial cells (Johnson et al. 2009). Micro-abrasion of the epithelial surfaces is believed to allow for this contact to be made (Schiller et al. 2010). Following this interaction there is a
conformational change in the virion capsid that allows cleavage of the N-terminal of the L2 capsid protein; this facilitates virus-epithelial interactions through an unknown receptor and subsequent internalisation of the virus (Doorbar 2005, Kines et al. 2009). Following internalisation of the virus particle it is transported in an endosome to the nucleus of the host cell where the genome is unloaded and the retained L1 capsid is degraded (Bergant Marusic et al. 2012, Doorbar et al. 2012, Schelhaas et al. 2012).
Figure 1.3 Phylogenetic tree of alpha human papillomavirus adapted from (Asgari et al. 2008)

Figure 1.4 Structure of HPV genes

Non coding Region
Promoter and enhancer

Late genes
L1 – major capsid protein
L2 – minor capsid protein
In the nucleus the genome is amplified to produce 50-100 copies of an episome (Maglennon et al. 2011). The E2 proteins are responsible for the initial viral genome amplification stage (Doorbar et al. 2012). The life cycle of the virus is clinically dependent on the differentiation of the epithelium. Basal host cells enter the cell cycle and proceed to differentiate to produce epithelial tissue in which the virus is able to amplify its genome to about 1,000 copies per cell (Stanley 2008). The E6 and E7 viral proteins ensure that the cell remains in the proliferative phase long enough for genome amplification and replication, which is followed by an E2 mediated down regulation of \( E6 \) and \( E7 \) and then production of viral structural proteins (Stern and Einstein. 2012).

The genome amplification phase is aided by the actions of the early proteins that have diverse targets and functions. The E7 protein performs its role by binding to the members of the retinoblastoma (Rb) protein family, which includes Rb, p105, p107 (involved in cell cycle control in the basal layers) and p130 (involved in cell cycle control in the mid epithelial region) (Roman 2006, Barrow-Laing et al. 2010). The high risk and low risk HPVs bind to this family of proteins with higher and lower affinity respectively (Felsani et al. 2006). Similarly, the E6 HPV proteins of both high and low risk types inactivate p53 protein, which is involved in cell cycle control, by a process involving ubiquitination and proteasome degradation (Fu et al. 2010, Pim and Banks 2010). Also the E5 protein contributes to genome amplification by interfering with apoptosis (Kabsch et al. 2004) and stabilisation of the epidermal growth factor receptor (EGFR) (Pim et al. 1992). For the low risk HPV types the functions of the E6 and E7 proteins at the basal layers still need further studies as their roles remain uncertain (Doorbar et al. 2012).

The synthesis of the late proteins is heralded by an increase in the E2 levels (Ozbun and Meyers 1998) which leads to the production of viral structural proteins L1 and L2 and assembly of an icosahedral capsid in the nucleus of the host (Day et al. 1998). The progression to cancer is associated with the integration of viral genome in the host genome and loss of regulatory \( E1 \) and \( E2 \) genes which allows for the uncontrolled expression of the \( E6 \) and \( E7 \) genes (Jeon et al. 1995, Jeon and Lambert 1995). However, this does not explain the observation of exclusive HPV 16 episomes in some cervical cancers (Matsukura et al. 1989, Vinokurova et al. 2008). Alternatively, Vinokurova and Doeberitz analysed the methylation pattern of the
HPV16 upstream regulatory region (URR) during squamous epithelial differentiation and neoplastic transformation and analyzed how shifts in the HPV URR methylome may affect viral gene expression and replication. Overexpression was associated with methylation of the distal E2 binding site 1 leading to hyper-activation of the HPV 16 URR (Vinokurova and von Knebel Doeberitz 2011). This might explain the loss of E2 regulatory function on E6 and E7. Either way the resulting genomic instability provides an opportunity for the generation of immortalized and transformed cancer cells (Doorbar et al. 2012).

Available methods identifying HPV include in-situ hybridisation (ISH), and/or polymerase chain reaction (PCR) to detect viral genes L1, E6 and E7 (Smeets et al. 2007, Schache et al. 2011, Bishop et al. 2012, Lewis et al. 2012) and/or detect biomarkers or cellular changes associated with papillomavirus induced cancers or infections (Moscicki et al. 2012, Chernock et al. 2013).

1.2.3 Human papillomavirus detection: current methods and challenges

The HPV cannot be cultured and thus relies on molecular techniques for accurate detection of type specific identification (Abreu et al. 2012). HPV detection in OPSCC relies on methods developed for cervical cancers, which can be summarised under four broad groups: 1) nucleic hybridisation, 2) nucleic acid amplification, 3) signal amplification and 4) detection of surrogate markers (protein expression). These methods succeed in identifying and typing HPV, in cervical cancers. As cervical cancer have been mostly linked to HPV the issue of demonstrating HPV oncogenesis in cervical cancer is not required. However, in OPSCC with the different aetiological factors having divergent prognosis, HPV detection and oncogenesis potential are important.

In OPSCC the common detection methods detect the viral nucleic materials and/or proteins associated with viral infections (p16 IHC). Nucleic acid detection methods for OPSCC include (but are not limited to) nucleic acid hybridisation (in-situ hybridisation, dot blot hybridisation, southern blot) and/or nucleic acid amplification assays (PCR, real time PCR [RT-PCR]). Nucleic acid (DNA or RNA) hybridisation methods involve the use of labelled nucleic acids hybridised to HPV genes in-situ. As detection depends on the copy number of the genes present, the
method is the least sensitive for infection/cancers with low viral presence (Villa 2006). In nucleic acid amplification methods, the target is amplified and detected during the procedure (real time) or at the end of the procedure. This method suffers from being overly sensitive.

ISH is a molecular biology technique that allows for the hybridisation of molecular probes to their complementary strands. The probes include double-stranded DNA, single stranded DNA, RNA and synthetic oligonucleotides. The probes are labelled using radioactive ($^{32}$P, $^{35}$S and $^3$H) and non-radioactive dyes (biotin, digoxigenin and fluorescence [FISH]). In the context of HPV detection, a cocktail of probes detecting certain high risk types are used. ISH of HPV DNA detection has been used for identifying episomal versus integrated patterns. As DNA detection gives no information about whether there is transcriptionally active disease, other types of ISH can be used to detect high risk HPV RNA (Ukpo et al. 2011, Bishop et al. 2012). These chromogenic and or fluorescence approaches in ISH are mostly limited by the scoring solutions used and relative insensitivity for humans. Automated methods although available are better deployed for high throughput methods. ISH which is more specific than sensitive should ideally be deployed as confirmatory and is not really a screening test.

PCR is a very sensitive method of amplifying targets for detection. It involves repeated heating and cooling cycles to amplify nucleic acids. It exploits the use of thermo stable DNA polymerase enzymes to elongate a pair of specific oligonucleotides flanking the target region. As repeated heating and cooling cycles progress, generated replicates can be detected real time or post cycling. Theoretically this method is capable of generating over a billion copies from a single strand of DNA or RNA (Zaravinos et al. 2009). In the context of HPV and OPSCC, this becomes important when limited clinical materials are available or a screening method is needed to delineate positive from the definitely negative HPV OPSCC. However, PCR can be limited by its sensitivity, in addition, with co-infection of HPV types there may be competition between the different HPV types for the available reagents (Abreu et al. 2012). Furthermore, the current use of consensus primers for detection of L1 may be limited by L1 loss or absence in OPSCC, yielding false negative result. The use of PCR for detecting transcripts of the HPV oncogenes ($E6$ and/or $E7$) has been described (Agoston et al. 2010). However, in the
context of multiple infections the requirement for a cocktail of type specific primers with redundancies can present problems.

p16 is a cyclin dependent kinase involved in cell cycle regulation that is related to pRB in a negative feedback loop. HPV E7 functional inactivation of pRB abrogates this relationship and results in up regulation of p16 (Dyson et al. 1989, Kamb 1994, Nobori et al. 1994). IHC detection of p16 is used as a surrogate marker of HPV induced oncogenesis. However, p16 upregulation/absence in OPSCC does not always correlate with HPV positivity/negativity. Mutations in CDKN2A (p16) and or other inter-related factors (E2F, RB and CDK4) have been described (Nevins 1998). In the context of HPV positive and negative OPSCC others have reported that p16 is sensitive but has poor specificity for OPSCC (Seiwert et al. 2015). Others have observed similar molecular profiles between p16 positive/HPV DNA negative and HPV negative patients (Cancer Genome Atlas Research et al. 2013, Rietbergen et al. 2014). This suggests that p16 is at best a surrogate marker for HPV infection and should preferably be used in combination with other detection methods.

The limitations in the methods described so far suggest that better detection methods are needed in OPSCC. It is now apparent that HPV positive and negative OPSCC are two different cancer types. This is evident by the significant differences in patient characteristics, tumour biology, tumour differentiation and survival features exhibited by patients with HPV driven OPSCC. HPV positive patients tend to be younger males, with basaloid and mostly poorly differentiated tumours who respond favourably to available treatment options. This makes the identification of these patients important not only for proper diagnosis but also for alternative treatment approaches with comparatively less associated morbidity. The prognostic differences in OPSCC require that there is an accurate HPV detection methods especially if this is likely to predicate treatment reduction.

1.3 Prognosis in oropharyngeal cancers

The prognostic classification of cancers can be related to the histo-pathological and specific molecular features and can lead to selection of different treatment options for the particular cancer patient. Common histo-pathological features include: the location, phenotype, size and spread (stage) and grade (differentiation) of the cancers. The list of molecular features are ever expanding; largely due to the
introduction of biomarkers identifying particular molecular changes such as hypoxia, angiogenesis, immune system activation, metastasis, DNA repair, etc. In OPSCC at present, it is unclear if viral infection specifically confers a prognostic advantage to HPV positive cancers.

Historically in OPSCC, survival rates have been analysed using the American Joint Committee on Cancer (AJCC) staging system. Overall 5-year survival rates have remained around 60% (Schlecht 2005, Ragin and Taioli 2007). However, recently it has emerged that HPV status is the strongest independent prognostic marker in OPSCC surpassing the staging classification (Fischer et al. 2010) and is irrespective of the treatment modality (Fischer et al. 2010). A meta-analysis by Ragin et al reported that patients with HPV positive OPSCC had a 28% reduced risk of death, and a lower risk of recurrence in comparison with HPV negative OPSCC (Ragin and Taioli 2007). Similarly, a recent meta-analysis of 42 studies by O’Rorke et al supports this position. Patients with HPV-positive HNSCC had a 54% better overall survival compared to HPV negative patients with a HR 0.46. The pooled HRs for tonsillar cancer and OPSCC were 0.50 and 0.47 (respectively. The pooled HR for disease specific survival was 0.28 and similar effect sizes were found irrespective of the adjustment for confounders (O’Rorke et al. 2012). It is now established that HPV positive HNSCC have a better prognosis than HPV negative disease as there is strong evidence supporting this observation (Table 1.3). However, factors responsible for this improved survival have not been clearly elucidated.

The mechanisms underlying the improved outcome of HPV positive OPSCC and how they may relate to other clinico-pathological/molecular factors are not understood. Two factors which might be responsible for the differences in survival are differential radiosensitivity, tumour hypoxia and immune system activation. Evaluating these mechanisms across a panel of OPSCC tumours may identify common factors related to prognosis in both HPV positive and negative OPSCC patients.
Table 1.3 Selected studies showing HPV positivity is associated with a good prognosis in OPSCC

<table>
<thead>
<tr>
<th>Study Reference</th>
<th>Patient No</th>
<th>HPV detection</th>
<th>Follow up time (months)</th>
<th>End point</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Al-Swahab et al. 2010)</td>
<td>274</td>
<td>HPV DNA p16 IHC</td>
<td>60</td>
<td>OS</td>
<td>OS</td>
</tr>
<tr>
<td>(Fischer et al. 2010)</td>
<td>102</td>
<td>p16 IHC</td>
<td>60</td>
<td>OS</td>
<td>0.0008</td>
</tr>
<tr>
<td>(Hoffmann et al. 2010)</td>
<td></td>
<td>HPV DNA p16 IHC</td>
<td>29</td>
<td>OS</td>
<td>0.012</td>
</tr>
<tr>
<td>(Li et al. 2003)</td>
<td>86</td>
<td>HPV DNA</td>
<td>48</td>
<td>DFS OS</td>
<td>0.012</td>
</tr>
<tr>
<td>(Shi et al. 2009)</td>
<td>111</td>
<td>HPV DNA p16 IHC</td>
<td>40</td>
<td>DFS OS DFS</td>
<td>0.0003</td>
</tr>
<tr>
<td>(Mellin et al. 2002)</td>
<td>22</td>
<td>HPV DNA</td>
<td>22</td>
<td>DSS</td>
<td>0.09</td>
</tr>
<tr>
<td>(Kuo et al. 2008)</td>
<td>92</td>
<td>HPV DNA p16 IHC</td>
<td>48</td>
<td>OS</td>
<td>0.004</td>
</tr>
<tr>
<td>(Rischin et al. 2010)</td>
<td>206</td>
<td>p16 IHC</td>
<td>48</td>
<td>FFS OS</td>
<td>0.003</td>
</tr>
<tr>
<td>(Charfi et al. 2008)</td>
<td>52</td>
<td>HPV DNA</td>
<td>60</td>
<td>OS</td>
<td>0.023</td>
</tr>
<tr>
<td>(Reimers et al. 2007)</td>
<td>106</td>
<td>p16 IHC</td>
<td>60</td>
<td>DFS</td>
<td>0.009</td>
</tr>
<tr>
<td>(Licitra et al. 2006)</td>
<td>90</td>
<td>HPV DNA p16 IHC</td>
<td>60</td>
<td>OS LRC</td>
<td>0.0018</td>
</tr>
<tr>
<td>(Rotnaglova et al. 2011)</td>
<td>109</td>
<td>E6mRNA E7mRNA</td>
<td>60</td>
<td>OS OS</td>
<td>0.004</td>
</tr>
<tr>
<td>(Ukpo et al. 2011)</td>
<td>211</td>
<td>HPV DNA p16 IHC</td>
<td>36</td>
<td>OS OS</td>
<td>0.03</td>
</tr>
<tr>
<td>(Heath et al. 2012)</td>
<td>83</td>
<td>HPV DNA p16 IHC</td>
<td>36</td>
<td>OS DFS OS DFS</td>
<td>0.03</td>
</tr>
<tr>
<td>(Young et al. 2011)</td>
<td>212</td>
<td>p16 IHC</td>
<td>36</td>
<td>DFS OS</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(Snietura et al. 2010)</td>
<td>59</td>
<td>HPV DNA p16 IHC</td>
<td>60</td>
<td>LRC</td>
<td>0.055</td>
</tr>
<tr>
<td>(Nassman et al 2013)</td>
<td>290</td>
<td>HPV DNA p16 IHC</td>
<td>36</td>
<td>DFS OS</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(Thibaudet al 2013)</td>
<td>255</td>
<td>HPV DNA p16 IHC</td>
<td>25</td>
<td>DFS OS</td>
<td>0.001</td>
</tr>
<tr>
<td>(Cerezo et al 2014)</td>
<td>102</td>
<td>p16 IHC</td>
<td>36</td>
<td>OS LRC DFS</td>
<td>0.05</td>
</tr>
<tr>
<td>(Heiduschka et al 2015)</td>
<td>63</td>
<td>HPV DNA p16 IHC</td>
<td>24</td>
<td>OS</td>
<td>0.04</td>
</tr>
</tbody>
</table>

OS = overall survival, DFS = disease free survival, LRC = loco regional control, DSS = disease specific survival. Studies included had greater than 20 months follow up with HPV detection by either p16 IHC and/or HPV DNA and/or HPV DNA ISH and/or HPVE6/E7 mRNA
1.4 HPV positive versus HPV negative OPSCC

As described above HPV positive and negative OPSCC are different cancer types originating in the same anatomical space. Risk factor and clinicopathological profiles differ in both groups. Several authors are of the opinion that HPV positive OPSCC should be regarded as a distinct disease entity (Dahlstrom et al. 2008, Gillison et al. 2008). This position is strengthened when several clinico-pathological features are compared. The typical patient with HPV positive OPSCC tends to be young (<50 years), a non-smoker / non-drinker (Dahlstrom et al. 2008, Gillison et al. 2008), there may be a history of oral sex and/or a history of multiple sexual partners (Gillison et al. 2008). There are histological differences between HPV positive and HPV negative OPSCC. Several authors report that HPV positive OPSCC are predominantly poorly differentiated non-keratinising squamous cell carcinomas exhibiting basaloid morphology while HPV negative OPSCC tend to be moderately differentiated and keratinised (Wilczynski et al. 1998, Gillison et al. 2000).

Differences between HPV positive and negative OPSCC are summarised in the table 1.4 below.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HPV positive</th>
<th>HPV negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatomy</td>
<td>Mostly tonsil and tongue base sites</td>
<td>No specific site</td>
</tr>
<tr>
<td>Persons at risk</td>
<td>Young non-smoking/non-drinking men</td>
<td>Older smoking/drinking men</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>Increasing incidence</td>
<td>Decreasing incidence</td>
</tr>
<tr>
<td>Risk factors</td>
<td>Number or oral sex partner, marijuana use</td>
<td>Smoking, alcohol and poor oral hygiene</td>
</tr>
<tr>
<td>Pathology</td>
<td>Poorly differentiated</td>
<td>Well/moderately differentiated</td>
</tr>
<tr>
<td>Mutational profile</td>
<td>Less mutation (approximately half of HPV negative)</td>
<td>More mutation</td>
</tr>
<tr>
<td>Biological profile</td>
<td>Occasional chromosomal loss</td>
<td>Gross deletion of large chromosomal regions</td>
</tr>
<tr>
<td>Epigenetics</td>
<td>CCNA1, GRB7, SYBL1, TIMP3, SFRP4, CDH11, JAK3, TUSC3, RUNX1T1, TCF21, IRX4, GATA4 and GFRA1</td>
<td>RASSF1, STAT5a, ALDHIA2, OSR2, SPDEF, MGMT, ESR2 and HSD17B12</td>
</tr>
</tbody>
</table>

Table 1.4 HPV positive versus negative OPSCC
1.5 Clinical presentation and treatment approaches

The most important factor determining the treatment at present is the stage of the disease (Marur and Forastiere 2008). One third of patients will present with early stage disease (Leemans et al. 2011). In early stage disease, patients may present with a history of sore-throat (unresponsive to antibiotics), chronic dysphagia, persistent odynophagia and otalgia (Marur and Forastiere 2008). Physical examination should include palpation of the regional lymph nodes which may be the only sign detected in some cases. This is followed by investigative procedures like endoscopy (nasopharyngolaryngoscopy) which helps to visualise/biopsy the tumours, others investigations like computed axial tomography (CT) and positron emission tomography (PET) help to determine the spread/extent of the diseases. HPV positive disease is reported to present with higher nodal disease at presentation (Gillison et al. 2000, Dayyani et al. 2010, Heath et al. 2012). OPSCCs are staged using the American joint committee on cancer staging guidelines (Table 4). This stages tumours based on size (T), extent of spread to the lymph nodes (N), and presence of metastasis (M). Presently staging is the single most important factor determining treatment (Forastiere et al. 2001, Marur and Forastiere 2008). Recent epidemiologic trends have not changed the management of OPSCC. Treatment options are similar to other head and neck cancers sites. Treatment is mainly directed towards achieving loco-regional control (primary metastasis is rare) and preserving organ function (Overgaard 2011).
Table 1.5  The American joint committee on cancer (AJCC) staging of cancers of the oropharynx

<table>
<thead>
<tr>
<th>Tumour (T)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Tumour 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour more than 2 cm but not more than 4 cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour more than 4 cm in greatest dimension or extension to lingual surface of epiglottis</td>
</tr>
<tr>
<td>T4a</td>
<td>Moderately advanced local disease</td>
</tr>
<tr>
<td>T4b</td>
<td>Tumour invades the larynx, extrinsic muscle of the tongue, medial pterygoid, hard palate or mandible</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional Lymph node (N)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph node cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension, or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension or in bilateral contralateral lymph nodes, none more than 6 cm in greatest dimension.</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastasis in a single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2b</td>
<td>Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2c</td>
<td>Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in a lymph node more than 6 cm in greatest dimension</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant metastasis (M)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

### 1.5.1 Early stage disease (T1 and T2 N0 M0)

The Scottish intercollegiate guidelines network (SIGN), national comprehensive cancer network (NCCN) and the ENT UK multidisciplinary management guidelines advocate the use of primary surgery and/or radical radiotherapy. Surgery for early stage disease could either be an open surgery followed by reconstruction with a pedicled flap (Haigentz et al. 2009) or could be via a trans oral approach using laser...
(TLM) (Grant et al. 2006, Rich et al. 2009) or robots (TORS) (O'Malley et al. 2006). Occasionally primary surgery is followed by dissection of regional lymph nodes either as an elective or therapeutic procedure. Reviews of this practice is mixed, a meta-analysis reported that there is insufficient evidence to conclude that elective nodal dissections improved overall survival (Bessell et al. 2011). However, neck dissection of the regional lymph nodes also allows for accurate pathological staging (Forastiere et al. 2001). Radical radiotherapy is often also with fractionation schedules altered dependent on presence or absence of lymph node involvement (Haigentz et al. 2009).

1.5.2 Locally advanced disease

About 60% of the patients will present with locally advanced disease (Forastiere et al. 2001, Haigentz et al. 2009). Treatment involves a multimodal approach combining surgery, chemotherapy, radiotherapy and recently biological therapy. Radiotherapy is an important treatment modality in these patients and as such radiobiological principles were used to develop altered fractionation regimens to improve treatment and loco-regional control while limiting toxicity (Krstevska 2009). Chemotherapy can be administered before (Induction) during (concomitant) and after (adjuvant) radiotherapy. Induction chemotherapy using: taxanes, platinum compounds, and fluorouracil (TPF) has been shown to be effective high risk patients (Posner et al. 2007, Vermorken et al. 2007, Paccagnella et al. 2010). In addition, biological therapy has been investigated in locally advanced HNSCC. In a phase III trial Bonner et al investigated cetuximab in 211 patients. They reported that median duration of loco-regional control (LRC) improved from 14.9 to 24.4 months with the addition of cetuximab to radiotherapy (HR, 0.68; P=0.005). Median duration of overall survival (OS) improved from 29.3 to 49 months (HR,0.74; P=0.03). Also, there was a significant prolonged progression-free survival (HR,0.70;P=0.006) (Bonner et al. 2006). Updated results reported 5-year overall survival rates of 45.6% in the cetuximab-plus-radiotherapy group and 36.4% in the radiotherapy-alone group (Bonner et al. 2010). However, preliminary results from the recently concluded RTOG 0522 trial investigating cetuximab plus chemoradiation in locally advanced head and neck cancers indicate that there’s no added benefit to this combination (Shin and Khuri 2011) but final results are awaited.
1.5.3 Recurrent and metastatic disease

Following treatment for locally invasive disease some patients go on to develop recurrent local and or metastatic disease (Shin and Khuri 2011). In addition, 10% of patients present with metastatic disease at diagnosis (Jemal et al. 2009). Patients in this category have a poor prognosis with a median survival of about 6 to 8 months (Shin and Khuri 2011). Historic treatment involved the use of salvage surgery followed by reirradiation (Shin and Khuri 2011). The EXTREME (erbitux in the treatment of recurrent and metastatic) phase III trial investigated the use of the biological agent cetuximab. In the trial 420 patients with were randomised to receive chemotherapy (cisplatin or carboplatin plus 5-flourouracil) alone or plus cetuximab. Their finding suggested that the addition of cetuximab improved median overall survival from 7.4 to 10.1 months (HR, 0.80; 95% CI, 0.64 to 0.99; P=0.04), prolonged progression free from survival 3.3 to 5.6 months (HR, 0.54; P<0.001) and also increased the response rate from 20% to 36% (P<0.001) (Vermorken et al. 2008). An earlier study comparing cisplatin plus cetuximab vs. a placebo in patients with recurrent or metastatic HNSCC did not report similar increases in overall survival and progression free survival. However, there was a significant improvement in response rate (P=0.03) (Burtness et al. 2005, Strojan et al. 2011). In November 2011, the food and drug administration (FDA) approved the use of cetuximab (erbitux) in combination with platinum based agents plus 5-flourouracil for the first line treatment of recurrent loco-regional disease and or metastatic HNSCC (FDA 2011).

1.5.4 Treatment of HPV positive versus negative OPSCC

The current standards of treatment described above applies to HPV positive and negative patients and is solely dependent of disease staging. Available evidence suggests that staging is not prognostically significant in HPV positive disease (Oguejiofor et al. 2013). In addition the predominance HPV OPSCC in no smoking young men with comparable better outcome following treatment encourages less toxic treatments. Treatment de-escalation has been suggested, there are several on-going trials evaluating treatment de-escalation approaches (Elrefaey et al. 2014). Therefore the most important factor in this trials is the proper HPV classification of these patients and subsequent therapeutic groupings. Also within the larger
aetiological groupings there will be patients who will have prognostic stratification different from the larger subgroups. Therefore biomarkers are needed to stratify these patients after proper HPV classification.

Difference in the activation of the immune system can be exploited for therapy and biomarker development. At present two prophylactic HPV vaccines are available for prevention of HPV infections. These vaccines are delivered by a series of intramuscular injections which provide cover for up between 6-9years (dependent vaccine valency) (Stanley 2010). At present the Advisory Committee on Immunisation Practices (ACIP) and the Centre for Disease Control (CDC) recommends vaccination of both young boy and girls and high risk groups: adolescent men, men who have sex with men (MSM), lactating women and women with equivocal pap test results (Markowitz et al. 2007). Established infections, pre-cancer and or cancer would require the development of therapeutic vaccination strategies. The foregoing therefore suggests that the importance of the immune system in the development of HPV positive OPSCC. In addition the characteristics of the immune micro-environment can be used as a biomarker for prognostic stratification in both HPV positive and negative groups.

1.6 The immune system

The immune system is a biological network of cells and molecules, which work collectively to protect multicellular organisms from pathogenic organisms. The actions of the immune system have been elucidated over the last century, are generally divided into recognition and response, and are mediated by innate and adaptive immune pathways (Figure 1.5).
### 1.6.1 Innate immune response

The innate immune protection begins from the anatomical barriers, which are the first line of defence against invading organisms; these barriers are usually several layers thick (skin, cervical, gut, bladder epithelium). The inherent differences in metabolism and structure of invading organisms are recognised by the innate immune system as dangerous (Akira and Hemmi 2003). These surveillance mechanisms recognise pathogen-associated molecular patterns (PAMP) (Janeway and Medzhitov 2002), which are sensed by the host immune cells using pattern recognition receptors (PRR) (Takeuchi and Akira 2010) and/or pathogen inflicted damage or stress induced pathways (Iwasaki 2012). Following recognition, the immune system activates a cascade of inflammatory responses which are mediated by soluble (cytokines, chemokines, and interferons) and cellular mediators to facilitate pathogen eradication (Kumar et al. 2011). Furthermore, the innate immune system is the key messenger leading to activation of specific adaptive immune responses.
1.6.1.1 Innate recognition mechanisms

The innate immune system involves the cooperation of a number of cells that include: macrophages, neutrophils, dendritic cells (DC), basophils, monocytes, natural killer (NK) cells and mast cells. These cells continuously survey tissues and recognise any disturbance of homeostasis (de Visser et al. 2006).

The innate immune system recognises PAMPs using PRRs; several of the PRRs have been described including the toll like receptors (TLR) (Lemaitre et al. 1996). There are 10 TLR families in humans, which detect different ligands intracellularly or at the plasma membrane (Iwasaki and Medzhitov 2004). TLR 1, 2, 4, 5 and 6 are located on the plasma membrane and recognise PAMPs derived from bacteria, fungi and protozoa. TLR 3, 7, 8 and 9 are located in the intracellular compartment and recognise nucleic acids from bacteria and viruses (Kumar et al. 2011). Following activation of a TLR by PAMP, there is recruitment of downstream adaptor molecules (MyD88, TRIF, TIRAP and TRAM), which activate transcription factors involved in the production of pro-inflammatory mediators (Kumar et al. 2011). Other PRRs that have been described include: RIG-I-like (RLR), NOD-like (NLR) and DNA receptors.

The RLR family consist of three members (RIG-I, MDA5 and LGP2) which sense and recognise RNA from RNA viruses present in the cytoplasm of infected cells. They subsequently induce an inflammatory response by producing inflammatory mediators to recruit immune cells to the site of infection (Wilkins and Gale 2010). Similarly, the NLR sense PAMP ligands, present in the cytoplasm and lead to recruitment of inflammatory mediators. NLRs can also activate an inflammasome, which cleaves several caspases involved in the production of inflammatory mediators or initiation of cell death (Kumar et al. 2011). The NLRs are able to recognise a wide range of PAMPs from different organisms. Furthermore, reports suggest immune cells are able to respond to DNA PAMPs by a mechanism which requires TBK1/IKKi (DLMI and ZBP1) as a cytoplasmic sensor (Kumar et al. 2011). Complementing the recognition of viral metabolic and structural features, innate immune cells (macrophages and DC) are able to recognise perturbation of homeostasis caused by viral infections (Iwasaki 2012). Using the NLR model, initial signal is transduced by the TLR which leads to the up regulation of NLRp3 and a second signal occurs as a result of perturbations of membranes by viral activities such as entry, fusion, budding and replication (Sutterwala et al. 2007). These signals
lead to activation of innate immune cells and pyroptosis of infected cells (Iwasaki 2012).

Humoral components of the innate immune system such as: mannose-binding protein (MBP) and other collectins, lipopolysaccharide binding protein (LBP), CD14, C-reactive proteins and complements are able to recognise invading pathogens present in the extracellular environment (Beutler 2004). Mannose-binding proteins recognise terminal mannosyl residues present on the surface of microbes and can activate the complement system via the mannose-binding protein-associated serine protease (MASP) pathway (Matsushita et al. 1998).

1.6.1.2 Innate effector mechanisms
Following adequate sensing by a recognition pathway, the cell-based effector mechanisms of the innate immune response secrete cytokines that act on other immune cells and/or generate a systemic response to inflammation aimed at attracting other inflammatory cells to the site to eliminate pathogens and/or infected cells by phagocytosis (Beutler 2004). In the phagosome, these microbes are eliminated using a myriad of cytokines, chemokines, histamines, metalloproteinase (MMP), reactive oxygen species (ROS) generated by NADPH phagosome oxidase (phox), and reactive nitrogen species (RNS) generated by inducible nitric oxide synthetase (iNOS) (Beutler 2004).

The innate immune effector cells can target cancer cells directly. NK cells have two types of functional receptors on the cell membrane with inhibitory and stimulatory functions (Yokoyama and Plougastel 2003). Stimulatory signals from the appropriate ligand mediate apoptosis in target cells, e.g. the UL16 binding protein, MHC class-I- chain related protein A or B (Bauer et al. 1999). Signals from these ligands activate the NK cells to secrete interferon γ (IFN-γ), perforin and granzyme and other inflammatory cytokines (Liu and Zeng 2012). The stimulatory effects are counteracted by the presence of an inhibitory signal through killer-cell immunoglobulin like receptor (KIR) (Long 1998). The binding of ligand to the inhibitory receptors supresses the cytotoxic activity of NK cells. Also seen on the cell membrane of NK cells are tumour necrosis factor (TNF) ligands which induce apoptosis in tumour cells expressing TNF receptors (Liu and Zeng 2012).

Macrophages and dendritic cells recognise molecules associated with apoptosis, e.g. lipid phosphatidylserine (PS), oxidised PS calireticulin using scavenger
receptors (SR-A, CLA-1, CD36, CD68, LOX-1 and stabilin-2), and T-cell immunoglobulin mucin (TIM) proteins. Recognition and phagocytosis of apoptotic tumour cells are achieved without stimulating significant pro-inflammatory cytokines, and this can favour induction of immune tolerance (Liu and Zeng 2012). Also antibody-dependent cell-mediated cytotoxicity is mediated by NK cells and macrophages (Nakamura et al. 2008, Nimmerjahn and Ravetch 2008). Furthermore, recruitment and activation of the adaptive immune mediators can be mediated by NK (Wang et al. 2005, Fan et al. 2006) and dendritic (Klechevsky et al. 2008) cells.

1.6.2 Adaptive immune system

The activation of the adaptive immune system is largely mediated through the activity of dendritic cells (DC), which are “professional antigen presenting cells”. DC reside at barrier sites where they can have close interaction with antigens (Iwasaki and Medzhitov 2010). Dendritic cells engulf self/non-self-proteins present in the environment and package them in the MHC class I or II molecules destined for the cell membrane. The activation of the innate immune system at these sites of danger (described above) leads to the activation and migration of a mature dendritic cell to the draining lymphoid tissue where they can interact with the T lymphocyte population (Dempsey et al. 2003). Different subsets of DCs have been described (Shortman and Liu 2002).

Antigen presentation of the different subsets leads to the activation of distinct lymphocyte phenotypic (T_H1 and T_H2) responses (Dempsey et al. 2003). The activation of specific T lymphocytes (CD4 or CD8) is mediated through the interaction of T cell receptors (TCR) with peptide presented by MHC class I/II molecules respectively (Iwasaki and Medzhitov 2010). CD4 T cells (helper T cells) are required for the optimum activation of CD8 T cells and also B cell responses. Plasticity in biological systems allows for adaptation to diversity in the environment. This is particularly important to maintain immunocompetence in adulthood following thymic involution. CD4+ T cell plasticity allows for production of heightened responses to specific antigens the CD4+ T cells (Caza and Landas 2015). Subset specification of naïve CD4+ T cells depends on innate signals, cytokine and chemokine profiles, while subsequent lineage specification requires specific transcription factors and speigenetic changes (Bluestone et al. 2009). Subset and
lineage specification is not absolute as CD4+ T cells are capable of adopting alternate fates (Caza and Landas 2015).

Naïve T cells require two signals to be activated, a TCR mediated signal and a co-stimulatory signal. The TCR are activated by the presence of peptides loaded onto the MHC molecules of the APC (Stone et al. 2009, Lichtenfels et al. 2012). Costimulatory signals are mediated by the interactions of CD28 proteins present on T cells and its B7 family of ligands expressed on activated APCs (Allison 1994, Lichtenfels et al. 2012). With TCR and CD28 activation there is proliferation and secretion of cytokines by T cells and in the absence of co-stimulatory signals the activated T cells become anergic and die (Sugamura et al. 2004). Activated T cells up-regulate CTLA-4 (Watts and DeBenedette 1999, Sharpe and Freeman 2002) and inhibitory programmed death-1 (PD-1) (Freeman et al. 2000, Nishimura and Honjo 2001), which bind to B7 ligands to down regulate T cell functions. Such inhibitory signals provide for a homeostatic balance between positive and negative immune functions (Parry et al. 2005). Targeting the CTLA-4 and PD-1 receptors has been suggested as a means of promoting anti-tumour immune actions (Phan et al. 2003, Parry et al. 2005).

Exogenous and endogenous signals associated with MHC class II or I activate CD4+ or CD8+ T cells respectively (Medzhitov and Janeway 1997, Chaplin 2010). The adaptive immune cells differ from the innate immune cells by their expression of somatically generated antigen-specific receptors on their cell surface. This expression allows for recognition of specific antigens and the generation of a robust immune action against these antigens (de Visser et al. 2006). The initial adaptive immune response is relatively slower as it requires the clonal expansion of lymphocytes specific to a particular antigen. However following the initial primary challenge, a subset of lymphocytes persists and are able to mount an anamnestic response to subsequent encounters to the antigen (de Visser et al. 2006, Goldszmid and Trinchieri 2012).

The elimination of self-reacting T cells in the thymus (Starr et al. 2003) and the requirement of co-stimulatory receptor activation prevents the action of T cells against self-peptide-MHC complexes. In addition a population of T cells with immune suppressive functions (T regulatory cells [Tregs]) adds another layer of protection against auto-immunity. Tregs are CD4+CD25+Foxp3+ expressing T cells
that are produced at central and peripheral sites. The generation of a Treg response depends on the co-operation of TCR signalling, cytokines (TGF-β and IL2) and the proliferation kinetics of T cells (Josefowicz et al. 2012). The Tregs disrupt the immune activating effects of transcription factors: nuclear factor of activated T cells (NFAT), acute myeloid leukemia-1/runt-related transcription factor 1 (AML1/Runx1), histone acetyl transferase/histone deacetyl transferase (HAT/HDAC) and NF-κB (Sakaguchi et al. 2008). These diverse transcription factors are critically involved in immune related signal transduction (Zap70 and Ptpn22), cell surface interactions (IL2ra, CTLA-4 and FasL), cytokine production (IL2 and IL4), metabolism (Pde3b) and others involved in a diverse range of immune functions. The role of Tregs in autoimmunity and lately cancers has been studied and reported on (Sakaguchi et al. 2008, Byrne et al. 2011).

1.6.3 Cancer immunology

1.6.3.1 Immune surveillance
Knowledge of the interactions of cancers and the preventive functions of the immune system has evolved over the last century. The cancer “immunosurveillance” hypothesis proposed that immune cells constantly survey host tissues and carcinogenesis occurs when these systems are overridden (Burnet 1964). Observations by Stutman suggested otherwise in his studies comparing 32 athymic “nude” and 43 normal mice following injection of 3-methylcholanthrene (MCA). He observed no difference in the incidence of tumours between both arms (18% and 17% respectively) (Stutman 1974, Stutman 1979). Improved technological advances allowed the revisiting of this concept (Smyth et al. 2001, Dunn et al. 2002). The use of genetically modified immune deficient mouse models led to the recognition of the role of immune surveillance mechanisms (Dighe et al. 1994).

Also, using population-based cancer registry records, Vajdic and Leeuwen studied the incidence of cancer amongst organ transplant recipients with iatrogenic immune suppression. They found an excess risk of developing virally induced cancers amongst recipients; however cancers not associated with a viral aetiology such as breast, prostate, ovarian, brain and testicular cancers showed no excess risk (Vajdic and van Leeuwen 2009). This finding suggests that the immune system might only be active against cancers with an infectious origin. Observations in animal models
suggest otherwise (Teng et al. 2008). Similarly, studies investigating IFN-γ (Shankaran et al. 2001) and perforin (Smyth et al. 2000) have shown a link between immune deficiency and the development of spontaneous, transplanted, chemical or viral induced cancers (Dunn et al. 2004, Chow et al. 2012). Studies in mouse models show that depletion of NK cells using anti-NK1.1 monoclonal antibody resulted in these mice developing 2 to 3 times more chemically induced cancers than control mice (Smyth et al. 2001). Also inadvertent transplant of a solid tumour via a donated kidney in an immune-deficient human led to reactivation of a dormant tumour (MacKie et al. 2003). These observations support the idea of the role of the immune system in preventing cancers.

1.6.3.2 Immuno-editing and immune escape

Emerging in recent times is the concept of “cancer immune editing” with the previous concept of immune surveillance representing an aspect of the relationship between cancers and the immune system (Dunn et al. 2004, Teng et al. 2008). The proponents describe three dynamic phases: elimination (surveillance), equilibrium and escape each characterising the complex interplay between the immune system and cancers. The cells of the adaptive and innate arms of the immune system have been linked to the immune surveillance (Dunn et al. 2004). Following incomplete elimination, cancer and immune cells may exist in a state of equilibrium where there is some tolerance to these cells by the immune system. Koebel et al describes this as an equilibrium phase (Koebel et al. 2007), which may be followed by two outcomes: a return to the elimination phase or “escape”. Escape is heralded by “cancer immunoediting” a process where less immunogenic phenotypes of the cancer cells are selected, which eventually leads to disease progression (Dunn et al. 2002).

Evidence for the immune-editing process is seen in studies on mice. Transplanted tumours from immune deficient mice were shown to be less successful in forming secondary tumours in immune competent host, while tumours from immune competent mice were more successful in both hosts (Kim et al. 2007, Teng et al. 2008, Hanahan and Weinberg 2011). This might suggest the participation of the immune system in selecting more or less immunogenic tumours in immune deficient or immune competent hosts respectively. Others have reported the frequent loss of HLA class I proteins in human tumour specimens (Algarra et al. 2000), and other components of this pathway (Dunn et al. 2004). Also implicated in immune escape
are immunosuppressive cytokines TGF-β and IL-10 (Khong and Restifo 2002, Shields et al. 2010, Yang et al. 2010) and immunosuppressive T cells (CD4+CD25+ regulatory T cells) or myeloid derived suppressor cells (MDSC) (Dunn et al. 2004, Ostrand-Rosenberg and Sinha 2009, Mougiakakos et al. 2010). In addition tumour cells may become resistant against apoptotic signals (Reed 1999). The concept of immune surveillance from early uncertainty is now firmly established as the data reflect a struggle between immune control and tumour evolution and escape.

1.6.3.3 Tumour promoting inflammation
The immune system, while controlling cancer emergence, can also act as a catalyst for its development through chronic inflammation. This inflammation which ensues with or without the immune editing process is known to promote the development of tumours. Chronic activation of the immune system leads to extensive tissue remodelling, angiogenesis (Grivennikov et al. 2010) and suppression of adaptive immune responses (de Visser et al. 2006). There is also evidence of studies reporting unfavourable outcomes in patients with infiltrating innate immune cells in breast cancer (Leek et al. 1999), melanoma (Ribatti et al. 2003) and adenocarcinoma of the lung (Imada et al. 2000). Furthermore, there are a number of chronic inflammatory conditions associated with cancer predisposition (Balkwill et al. 2005). In mouse models, chronic infiltration of innate immune cells in pre-malignant skin and cervical lesions infected by HPV 16 differs depending on the site of the lesion (van Kempen et al. 2002, Giraudo et al. 2004) (de Visser et al. 2006). Similarly, the link between chronic inflammation and cancer is strengthened by the fact that prolonged use of non-steroidal anti-inflammatory drugs has been shown to be protective against certain cancer types (colon, breast, prostate and lung) (Vendramini-Costa and Carvalho 2012).

1.6.3.4 Tumour promoting versus preventing inflammation
It is unlikely that there is an arbitrary transition point between protective and promoting immune response to tumours. The cancer immune editing theory recognises immune promotion of cancers as a failure of surveillance and reports suggest there is an overlap of this process (Chow et al. 2012). Also puzzling is the bi-functionality of cytokine and immune cells in cancer prevention and promotion. For example TGF-β has both tumour promoting and suppressive functions (Massague 2008), and so does IFN-γ (Zaidi et al. 2011). Secretion of IFN-γ by activated T cells leads to upregulation of MHC I and II on tumour cells leading to better efficient
antigen presentation (Freedman et al. 2000, Dunn et al. 2006). Further increasing this already complex picture is the seemingly different functions of cytokines in different tissue models (Chow et al. 2012). It is not known whether it is possible to distinguish a tumour promoting and a preventive inflammation. Investigators have studied the infiltration of several relevant immune cell types and compared this with patient survival.

1.7 Tumour infiltrating lymphocytes and cancers

Infiltration by immune cells is common in most cancer types, and their presence may be a result of a tumour preventing or promoting inflammation (Kim et al. 2007). A significant observation is that the presence of a predominant T lymphocyte repertoire is associated with a favourable prognosis in several cancer types (Pages et al. 2010), which may be indicative of a tumour preventing inflammation role. It is suggested that effector T cells infiltrate tumour sites as a result of tumour immunogenicity with the aim of eliminating and/or controlling/delaying the spread of these cancer cells. The adaptive immune response which is initiated at antigen presentation leads to expansion of the relevant lymphocytes and the activation of a cell or humoral mediated immune response. CD8+ cytotoxic T cells, which are key effector cells of the cell mediated adaptive immune response, are capable of killing tumour cells. Therefore, the presence of this subpopulation of lymphocyte is assumed to be indicative of preventive immune response to the tumour. However, there is a multiplicity of T cell types which provides both positive or negative effects on tumour growth depending on the subtype (differentiation) and/or their activation status.

In the landmark study by Galon et al on 959 patients with colorectal tumours who were followed up for 15 years, the investigators used several complementary methods to study immune infiltrating cells in colorectal cancers. They performed gene expression profiling on 75 of these tumours and observed that there were increased levels of genes associated with Th1 phenotype and effector T cell function in patients with absent metastatic features. Using flow cytometry on 39 fresh samples, they found increased markers of T cell adhesion, migration and differentiation and significant increases in early memory to effector memory T cells in patients without metastatic features (Pages et al. 2005). The study used tissue
microarrays to investigate the densities of TILs at the centre of the tumour and invasive margin. Increased immune cell densities at both sites were observed in patients with non-recurrent tumours. From this, the authors developed an immune cell classifier which had a prognostic value superior to, and independent of, the traditional TNM classification (Galon et al. 2006). This demonstrated that the relationship between prognosis and TILs is dependent on immune cell type and cells/mediators associated with inflammation.

There will always be patients with high TIL density who do have metastasis (as was reported in the study) and these tumours will likely have evoked an immune escape (Pages et al. 2010). The unknown is how long the immune activity has already controlled tumour growth prior to detection. Immune regulatory processes are critical to homeostasis as their importance in limiting anti-tumour immunity is becoming increasingly apparent.

Infiltration by a predominantly Tregs repertoire has been linked with adverse outcomes in ovarian (Curiel et al. 2004) and liver (Hiraoka et al. 2006) cancers, but favourable outcomes in others (Alvaro et al. 2005, Badoual et al. 2006, Carreras et al. 2006). These conflicting reports may be due to the functional orientation and/or location of the cells (Fridman et al. 2011) and/or tumour type under consideration. More importantly it is clear that many negative and positive immune populations/factors can integrate to influence control/outcome.

In a recent meta-analysis of TIL studies across different tumour types a prognostic survival advantage was reported for infiltration by CD8+ T cells for all endpoints: overall survival, disease-specific survival. Infiltration by CD4+ T cells improved overall survival only. Treg studies which used FoxP3+ alone as a selective marker showed no statistically significant association with prognosis (Gooden et al. 2011). This might be related to the improper classification of Tregs requiring CD4+CD25 co-positivity in addition of Foxp3 positivity (Feuerer et al. 2009). Foxp3 is a transcription factor expressed on other types of lymphoid, myeloid and epithelial cells (Devaud et al. 2014). Therefore the use of Foxp3 alone is insufficient to characterise the Treg population. Others compared the ratio of effector to Tregs, and a meta-analysis found a statistically significant association with overall survival based on CD8+/FoxP3 ratios (Gooden et al. 2011). Despite the results from the meta-analysis, there were variations reported amongst different studies and tumour
types indicating that results cannot be extrapolated from one tumour type to another. It is unclear if previous studies on TILs were affected by differences in methodology such as tissue staining protocols and/or fixative techniques.

### 1.7.1 Tumour infiltrating lymphocytes in head and neck cancer

The association between HPV and OPSCC is well established (see Section 1.3). It is now clearly established as an important risk factor for the development of OPSCC (Singhi and Westra 2010). Furthermore HPV positive OPSCC are reported to have favourable survival irrespective of treatment when compared to HPV negative OPSCC (Ang et al. 2010). The reasons for these observations remain unclear and may be related to multiple factors. Investigators reported a close relationship between HPV, tonsil and tongue base sites in the anatomical oropharynx. It is however unclear what factors underlie this relationship. As it is known that the oropharynx plays host to several structures of the immune system, these observations raise the following questions: 1) What mechanisms allow HPV to evade immune activity and persist at these immune rich sites and subsequently lead to the development of cancer? 2) Does the immune system have a role in limiting the spread and/or the response of HPV induced cancer cells to therapy?

Co-stimulatory inhibitory pathways have been suggested to be involved in providing a niche environment for oncogenesis. The interaction between programmed cell death protein 1 (PD1) on activated T-cells and its ligands PD-L1 and PD-L2 expressed on antigen presenting cells (APCs) have been described. The activation of this pathway will lead to the inhibition of T cell function, thereby promoting the persistence of infection. In their study investigating the role of PD-L1-PD1 pathway in OPSCC, Lyford-Pike et al reported that there is expression of immune inhibitory ligands PD-L1 at the reticulated epithelium of the deep crypts and not the surface epithelium of the tonsil of HPV/cancer naïve patients. They also reported a higher PD1 expression on CD8+ TILs and peripheral blood mononuclear cells in HPV positive cancers when compared with chronically inflamed tonsils. The authors suggested that the interaction between PD-L1 and PD1 might contribute to HPV immune evasion (Lyford-Pike et al. 2013).

Several studies have reported on the relationship between TILs and prognosis in HNSCC. In early studies by Synderman et al, 16 patients with HNSCC were
evaluated for T cell markers CD4 and CD8 using IHC and two colour fluorescence activated cell sorting (FACS). Patients with a low CD4/CD8 ratio (as a result of a higher CD8 T cell population) were less likely to develop tumour spread (Snyderman et al. 1989). However, assessing CD4 alone will not identify Tregs which are known to infiltrate and suppress immune activity in the tumour micro-environment of HNSCC (Strauss et al. 2007, Bergmann et al. 2008). However, others have reported favourable outcome with Treg infiltration (Wansom et al. 2012). A study on patients with oral squamous cell carcinoma reported that CD8+ TIL density correlated significantly with tumour size (P=0.003), lymph node metastasis (P<0.001) and clinical stage (P<0.001) (Ascierto et al. 2013). Similarly, Watanabe et al reported that lower infiltration of CD8+ T cells at tumour nest and stromal sites was associated with decreased survival, while increased stromal CD8+ T cells over the total Treg population did not (Watanabe et al 2013). This suggests that the localisation of TILs might be important when evaluating their prognostic effects in HNSCC.

Also pertinent is the presence/absence of other immune evading properties in HNSCC, e.g. expression of PD1 receptors in HNSCC. In a study on early stage tongue cancers, Katou et al reported that CD8+ TILs invading tumour nests had frequent expression of PD1 in contrast to adjacent normal stroma and early pre-cancerous lesions (lichen planus) (Katou et al. 2007). Similarly, others showed that PD-L1 expression by tumour associated fibroblasts was associated with a lower density of intratumoural CD8+ T cells (Ascierto et al. 2013). This emphasises the need to assess the functional status of TILs and surrounding stroma in tumour phenotypes. Furthermore, others suggest that the prognostic significance of TILs in HNSCC might be related to the stage of the disease (Distel et al. 2009) and the lymphocyte distribution in regional lymph nodes (Pretscher et al. 2009).

In recent times with the changing paradigm of OPSCC there is the desire to evaluate the differences or similarities between TILs in HPV positive versus HPV negative OPSCC. Particularly interesting is the role of potentially different tumour associated antigens which may/may not lead to the activation of a differing immune cell repertoire. Furthermore, this tumour site provides an opportunity to evaluate the diverging response (if any) of immune cells infiltrating viral and non-viral mediated
cancers. This opportunity was not available to early investigators of TILs in HNSCC.

In a recent study, Wansom et al. studied pre-treatment biopsy samples of 46 patients with advanced OPSCC for markers of TILs (CD8, CD4, CD68 and FoxP3) and analysed with respect to HPV status, EGFR expression, and patient outcome (Wansom et al. 2012). They reported increases in T cell infiltration associated with patient survival, but not related to HPV status. In addition reduced T cell infiltration was seen in tumours with higher EGFR expression; higher counts of CD4+ and CD8+ TIL in HPV-16 positive tumours, and higher levels of circulating CD8+ T lymphocytes in the peripheral blood of all patients, however, this did not correlate with the density of TILs (Wansom et al. 2012). This complex set of “conclusions” provides limited insight because there were only a few patients with advanced diseases. The latter are more likely to have developed proficient immune escape mechanisms with increasing disorganised architecture limiting immune activity. However, the work suggests that the immune system does play a role in patient survival in HPV positive OPSCC and that further detailed studies are necessary.

1.8 Human papillomavirus, radiosensitivity and hypoxia

The improved survival of HPV OPSCC following radiotherapy (Kong et al. 2009) may be due to a differential responses to radiation. Differences in intrinsic radiosensitivity and/or tumour hypoxia have been implicated (Kimple et al. 2013, Sorensen et al. 2013). Studies comparing the radiosensitivity of HPV positive and negative tumours have been largely done in cell lines or a few patients. In an observational cohort study on 10 HPV +ve patients matched with negative controls who then received intensity-modulated radiation therapy (IMRT), Chen et al observed a statistically significant difference in tumour control between both groups as the HPV +ve group had a more rapid rate of tumour regression (Chen et al. 2013). However, these differences seen might be due to other factors unrelated to radiosensitivity.

A study compared the intrinsic radiosensitivity of HPV positive and negative head and neck cancer cell lines. It reported that HPV positive HNCs were more radiosensitive (average surviving fraction at Gy [SF2] 0.22) than HPV negative (average SF2 0.59), which corresponded with a prolonged G2-M cell-cycle arrest.
and increased apoptosis following radiation exposure. When they compared the genomic differences between the positive and negative cell lines they observed significant increases in p53 in HPV+ (P<0.0001) but not HPV- cell lines. To confirm this observation they knocked down TP53 using siRNA and observed increased radiation resistance (Kimple et al. 2013). In another study, Sorensen et al compared the radiosensitivity of HPV positive and negative cell lines and their response to hypoxia. They observed that although HPV positive cells were more radiosensitive than HPV negative cells, they displayed the same relative radioresistance under hypoxia and the same relative radiosensitisation by nimorazole (Sorensen et al. 2013).

The result from these studies established that there are differences in the radiosensitivity of HPV positive and negative cell lines. As shown above, these differences are linked to the differences in gene mutations between both tumour types. It is not clear if the virus (HPV) or its proteins modulate radiosensitivity before or after oncogenesis. The answer to this question could have implications for the treatment of patients. Interestingly the above also suggests that hypoxia response is the same in HPV positive and negative OPSCC cell lines. It is pertinent to mention that hypoxic conditions reproduced in-vitro in cell lines may not mirror in-vivo conditions. The dynamic nature of hypoxia in tumours also limits direct extrapolation of the results from the cell line study to tumours in-situ. Notwithstanding, the observation of similar hypoxia responses in HPV positive and negative OPSCC may have implications for therapy for these cancers.

1.8.1 Tumour hypoxia

The radio-resistance of hypoxic tumours is linked to the role of oxygen in potentiating radiation damage of DNA. Oxygen reacts with the free radicals produced when ionising radiation interacts with biological molecules, thereby ensuring lasting damage to DNA (Brown 2000). Oxygen therefore is a potent chemical radiosensitizer because it is a highly electron-affinic molecule (Ward 1988). Early observations by Thomlinson and Gray inferred the existence of hypoxic regions in tumours (Thomlinson and Gray 1955). Presently, it is now established that hypoxia exists in solid tumours (Vaupel and Mayer 2007). It is thought to arise as a result of the imbalances in the supply and consumption of oxygen by the tumour
(Brown 2000, Vaupel and Mayer 2007). Several mechanisms have been suggested to account for the development of tumour hypoxia. The vascularisation of is insufficient for a rapidly growing tumour. This presents the opportunity for aberrancy and disorganisation of blood supply to the tumour in comparison to normal tissue (Rademakers et al. 2008). The blood vessels in the tumour are characterised by a) morphological and functional deformities, b) varying diameters across blood vessel length, c) changing distances between blood vessels and d) intercapillary distances beyond the diffusion range (200 μm) (Konerding et al. 1995, Wilson and Hay 2011). The prevailing background thus present opportunities for: 1) transient disruption of blood supply (acute or perfusion limited hypoxia) by internal (occlusion) or external (increasing interstitial pressure from “leaky” blood vessels) factors (Jain 2002, Isa et al. 2006, Vaupel and Mayer 2007) and/or 2) the deterioration of diffusion geometry present in the tumour micro-environment further increasing the diffusion distances (chronic or diffusion limited hypoxia) (Brown 2000, Vaupel and Mayer 2007). Furthermore, anaemia as a result of the tumour and/or therapy can also contribute to a decrease in the oxygen available to tumours (Vaupel and Mayer 2007). The distinction between acute or chronic hypoxia is arbitrary and mostly academic as both exist in parallel in different sites of the tumour (Dewhirst 1998). Similarly the exact transition point in oxygen partial pressure between normoxia and hypoxia is not known but the metabolic changes associated with hypoxia are evident.

1.8.2 Tumour response to hypoxia

As the concentration of oxygen decreases, ATP production decreases (Hockel and Vaupel 2001). With sustained periods of hypoxia, there is cellular dysfunction as a result of declining ATP concentration. The cell compensates by the up-regulation of genes involved in hypoxia response (Hockel and Vaupel 2001). The hypoxia inducible factor (HIF) -1 is central to this pathway. It is composed of 2 subunits (α and β) and performs its function by binding to hypoxia response elements (HREs) in multiple genes (Wang and Semenza 1993). Jiang et al report that HIF-1α expression and HIF1 DNA binding capabilities increases with decreasing oxygen concentrations and vice versa (Jiang et al. 1996). HIF-1β is expressed continuously in cells while HIF-1α is hydroxylated and degraded in the presence of oxygen via ubiquination involving the presence of von Hippel Lindau protein (Graeber et al. 1996). This
stepwise process involves the hydroxylation of HIF-1α at proline residue by proline hydroxylase domain (PHD) proteins (Kaelin and Ratcliff 2008).

Prolyl-hydroxylated HIF-1α is detected by VHL and subsequently degraded. Under hypoxic conditions, hydroxylation is prevented and HIF-1α becomes expressed continuously and accumulates and dimerises with HIF-1β. Stable HIF-1 (HIF dimer) migrates to the nucleus and initiates the transcription of “hypoxia genes” (Brown 2000, Kizaka-Kondoh and Konse-Nagasawa 2009). These genes are involved in a myriad of cellular function such as energy metabolism (e.g. GLUT 1, GLUT3), angiogenesis (e.g. VEGF, TGFβ3), cell survival (e.g. ADM, BCL2) and invasion (e.g. AMF, CATHD) (Semenza 2003, Kaelin 2008). These genes and others are used as markers of hypoxia. Hypoxic cell death is mediated via p53 dependent and independent pathways (Vaupel and Mayer 2007). However, sustained hypoxia and the ensuing genome and proteome modifications may also promote tumour progression (Vaupel and Mayer 2007), and predict for a more aggressive, treatment resistant tumour phenotype. A systematic review of 40 retrospective studies on HNSCC evaluating the expression of HIF-1 and its downstream effectors (CA-IX, VEGF, GLUT-1) demonstrates an adverse prognostic profile with high expression of these markers (Wegner et al. 2015). Therefore, hypoxia modification is a valid method to improve therapeutic responses in this cancer type.

Others have investigated the role of the HPV genes in modulating the hypoxia response. In their study, Guo et al investigated the relationship between E6 and HIF-1α in cervical cancer cell lines. They reported E6 interference in the association between HIF1A and VHL. In addition the HPV E6 gene was linked to tumour enhanced glycolysis, the so called “Warbug effect” (Guo et al. 2014). The study involved transfection and co-immunoprecipitation of myc-tagged E6 and HIF1A under normoxia and hypoxia in the human embryonic kidney 293 cell lines. Transduction and knock down experiments were also performed in CaSki cells. However, the use of a non “native” HPV cell line, and the possible knock down of other factors downstream of E6 might limit the relevance of the observations. In a study on tumour xenografts, Hanns et al observed fewer hypoxic areas in HPV positive tumours when compared to HPV negative tumours. A lower hypoxia gene response and a higher density of new blood vessels were observed in the HPV positive OPSCC (Hanns et al. 2015). In a phase I and II trial of chemo-radiation of
patients with stage III and IV HNSCC, Trinkaus et al using $[^{18}\text{F}]$-fluoromisonidazole positron emission tomography reported wide spread areas in HPV positive (74%) and negative (80%) tumours.

The inconsistencies from the studies above could reflect the dynamic nature of hypoxia markers in the different models. Ideally hypoxia should be studied using well validated markers in patient tumour samples. The potential importance of identifying hypoxic tumours in HPV positive and negative tumours lies in the potential for selecting patients for hypoxia modifiers with radiotherapy.

### 1.8.3 Hypoxic modification in HNSCC

A meta-analysis of 32 randomised trials of different hypoxia modifying strategies reported a significantly improved benefit in loco-regional control, disease specific survival, and to a lesser extent in overall survival. The risk of distant metastases was not significantly influenced but appeared to be reduced in patients treated with hypoxia modification. Radiation related late complications were not influenced by the overall use of hypoxia modifications. The results from this meta-analysis supports the role of hypoxia modifiers in HNSCC, with evidence to support the greater effectiveness of hypoxia modification is greater for patients with hypoxic tumours. (Overgaard 2011)

Hypoxia modification strategies have involved widespread strategies including but not limited to use of: hyperbaric oxygen, carbogen and nicotinamide, nitroimidazoles, and selective targeting of hypoxic cells using tirapazamine. Despite the success as demonstrated in the Overgaard meta-analysis these methods are yet to be translated into clinical practice in most countries mostly due to issues in selecting patients who will benefit most from this approaches. Recently, emphasis has been directed towards using markers and/or gene signatures for patient selection. This approach has recorded some successes. In their study Toustrup et al developed and validated a 15 gene signature in 323 patients which showed both prognostic and predictive impact (Toustrup et al. 2011). In collaboration with Oxford, Manchester developed a 26 gene signature (Winter et al. 2007, Buffa et al. 2010). Eustace et al showed that this 26 gene signature predicted benefit from hypoxia modification with carbogen and nicotinamide (ARCON) (Eustace et al. 2013). Results from prospective application of these signatures in clinical trials are awaited. The UK NIMRAD trial (NCT01950689) (A Randomised Placebo-controlled Trial of
Synchronous NIMorazole Versus RADiotherapy Alone in Patients With Locally Advanced Head and Neck Squamous Cell Carcinoma Not Suitable for Synchronous Chemotherapy or Cetuximab) currently ongoing will use the 26-gene hypoxia signature (Thomson et al. 2014).

Other markers of hypoxia include: oxygen tension (Horsman et al. 1994, Nordsmark 1996, Nordsmark et al. 2005), plasma osteopontin concentration (Le et al. 2003, Overgaard et al. 2005), pimonidazole binding (Kaanders et al. 2002), and IHC detection of proteins upregulated under hypoxia (HIF, CA-IX, GLUT-1 and VEGF) (Young and Moller 2010). Studies have investigated whether hypoxia modification of radiotherapy benefit patients with HPV positive and negative HNCs.

When patients were stratified according to HPV status, patients with HPV/p16 negative tumours were reported to benefit more from hypoxia modification (Lassen et al. 2010). Using a tumour xenograft model, Sørensen et al observed a decrease in the level of cell proliferation in the HPV positive models following irradiation which was not seen in the HPV negative tumours. This might explain the lack of benefit for hypoxia modification in HPV positive OPSCC (Sorensen et al. 2014). Notwithstanding, identification of hypoxic tumours can be used to identify poor prognostic tumours and this may be important in the context of treatment de-escalation in HPV positive OPSCC.

There are some drawbacks to hypoxia modification. There is marked heterogeneity in hypoxic regions both within and amongst tumours of similar histology (Tannock 2005). This is evidenced by the diversity and lack of correlation among different hypoxia assays within individual tumours (Nordsmark et al. 2007). This has made it difficult to study tumour hypoxia and as a result standardise treatment. There is a need for standardisation of treatments and equally of biomarkers that identify patients who will benefit from hypoxia modification.

**1.9 Immunohistochemistry**

Immunohistochemistry (IHC) is an important research and diagnostic tool which bridges the disciplines of immunology, histology and chemistry (D'Amico et al. 2009). It functions on the basis of the bridging of hydrophobic and electrostatic bonds between antigens and antibodies. These interactions are aided by the presence
of amino-acid side chains in antigens and the ionic difference between antibodies (Matos et al. 2010).

Antibodies are proteins of the immune system, they are made up of four polypeptides arranged in a Y-shaped structure. The prongs are the antigen binding site (Fab) with a variable structure, which determines its idioype. The tail (Fc) is a constant region which binds to complements and other immune cells in-vivo. In the context of IHC, antibodies are made by immunising animals with synthetic peptides or purified protein extracts. The generated antibodies are either polyclonal or monoclonal, which differ in their sensitivity and specificity. The antibodies form complexes with antigens (proteins under consideration). The detection of a the anigen antibody complex using visible marker (chromogen or flourophore) is the basis of IHC. The IHC procedure consists of several sequential steps which begin with tissue collection and preparation and ends with cover slipping of stained slides and examination.

Following extraction, the tissue is immediately “fixed” (formalin) to preserve tissue morphology. The tissue is then embedded in either paraffin or plastic resin to maintain its physical structure. Conversely, to study antigens which will not survive formalin fixation and paraffin embedding, the tissue could be fresh frozen in liquid nitrogen. Following tissue preparation thin sections can be taken immediately or at a later date for IHC staining. A general IHC protocol is described in Table 1.4 and can be summarised in two phases: 1) slide preparation and 2) interpretation and quantification.

IHC has evolved since it was first described by Marrack (Marrack 1934) and Coons (Sasai et al. 2007). Its evolution as a scientific tool has been influenced by development of enzyme linked antibodies (Nakane and Pierce 1966), and the development of secondary antibody detection using peroxidase-antiperoxidase (Sternberger et al. 1970) and/or alkaline phosphatase – anti-alkaline phosphatase (Mason et al. 1978). These development allowed for the independent reproduction of IHC methods across laboratories and institutions. Furthermore, the development of antigen recovery methods (Huang and Robinson 1976) and other antibody detection approaches (avidin-biotin-peroxidase) (Hsu et al. 1981) has allowed IHC to be used reliably as a clinical diagnostic tool.
Table 1.6 Summarised IHC protocol

<table>
<thead>
<tr>
<th>Steps</th>
<th>Aims</th>
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<tbody>
<tr>
<td>1. Tissue extraction, fixation and</td>
<td>To collect and fix sections using formaldehyde and embed in paraffin</td>
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<tr>
<td>embedding</td>
<td>to maintain tissue architecture</td>
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<tr>
<td>2. Tissue sectioning</td>
<td>To section FFPE samples into thin (4-5μm) sections using a microtome</td>
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<td></td>
<td>and mount on glass slides</td>
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<tr>
<td>3. Antigen Retrieval</td>
<td>To unshield the target antigens by removing paraffin using xylene;</td>
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<td></td>
<td>rehydrating of sections in graded concentration of alcohol; and</td>
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<td></td>
<td>reversing formalin bridges between proteins either using heat (heat-</td>
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<td></td>
<td>induced epitope retrieval; HIER) or enzymes (proteolytic-induced</td>
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<tr>
<td></td>
<td>epitope retrieval; PIER)</td>
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<tr>
<td>4. Blocking endogenous enzyme activity</td>
<td>To block endogenous enzymes which may interfere with target detection</td>
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<td></td>
<td>systems using peroxidases or phosphatases.</td>
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<tr>
<td>5. Background blocking</td>
<td>To prevent weak interaction between antibody and non-specific sites,</td>
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<td></td>
<td>the so called “background staining”</td>
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<tr>
<td>6. Antibody incubation and detection</td>
<td>To incubate antibodies with target antigens, antigen-antibody</td>
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<td></td>
<td>reactions are then detected using either a direct or indirect detection</td>
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<tr>
<td></td>
<td>systems. The antigen-antibody reaction can be labelled using either</td>
</tr>
<tr>
<td></td>
<td>a fluorescent or chromogenic marker</td>
</tr>
<tr>
<td>7. Counter staining</td>
<td>To provide a good contrast between antibodies stained vs. unstained</td>
</tr>
<tr>
<td></td>
<td>tissue</td>
</tr>
<tr>
<td>8. Cover slipping</td>
<td>To preserve the section for long term use in an appropriate mountant</td>
</tr>
</tbody>
</table>

IHC has its drawbacks, which may be related to: a) improper technique or result interpretation, and/or b) limitations compared with other experimental techniques. Technical issues could arise from any step leading up to the examination of the slides. Delay in tissue fixation may result in proteome changes (Gundisch et al. 2013), sections that are too thin or thick may result in a weak or poor antibody staining of the sections respectively (Yaziji and Barry 2006). Fixation with formaldehyde and prolonged storage may reduce the immune reactivity to antigens.
Jacob et al reported a decrease in the intensity of p53 and Bcl-2 expression when breast tissue sections were stored for between 2-12 weeks (Jacobs et al. 1996), but similar findings were not reported in a study 520 colorectal adenocarcinoma sections (Manne et al. 1997). However, available evidence suggests protein degradation does occur following sectioning (Bertheau et al. 1998), and ideally IHC should be performed as soon as possible following sectioning. The antigen retrieval step is also susceptible to variations in procedure and antigen location. Cytoplasmic antigens may require enzymatic retrieval and heat (D'Amico et al. 2009). Furthermore technical issues could arise from the use of less or more of antibodies leading to differences in expression of a marker between studies (O'Brien et al. 2005).

Although IHC remains similar to its initial principle described in the 1930s and 1940s it has evolved and continues to evolve. For example, the development of tissue microarrays (TMAs) allows for high-throughput evaluation of the expression of candidate disease-related genes or gene products simultaneously on hundreds of tissue samples (Kumar et al. 2004). In recent years, the number and quality of antibodies has improved to make IHC a robust and standardised method for studying tissue antigens across different tissue types.

1.9.1 Multiplex immunohistochemistry

Multiplex IHC simply describes the process of staining two or more antigens on a single slide. This technique is important in histological examination of tissue sections as it allows for visualisation of the density and topographical relationships between antigens. This technique is important in studying TILs in cancers where the presence of multiple antigens differentiates cell type and/or functional status. Also multiplex IHC saves time and conserves tissue, which is important in the increasing patient population and decreasing tissue size which characterise modern clinical practice.

There are several technical challenges limiting the application of multiplex IHC these includes: A) Antibody type - antibodies generated from the same animal model can lead to cross reactivity of their Immunoglobulin (Ig) subtype. To prevent this, sequential staining techniques are employed, separated by a free antibody removal step. B) Antibody shielding – the first antibody and a chromogen label may shield other antigens which are in close proximity or co-localised on the same structure. The titration of the antibody to a suitable concentration usually addresses this
problem. C) Spectral differentiation of co-localised chromogen/flourophore – the
unaided eye is unable to distinguish between co-localised chromogens with similar
spectral properties (van der Loos 2008). Previously, proponents of multiplex IHC
suggested selecting contrasting chromogens to provide contrasting spectra. However,
with the introduction of multispectral imaging this issue of metamerism is addressed
and the need for contrasting chromogen selection is circumvented.

From the above it is evident that the problems which previously limited the
implementation of multiplex IHC are being overcome. In the future, the use of
multiplex IHC as a histological technique will expand. However, at present there is a
need to standardise this methods for use across several tissue types. Pivotal to the
introduction of multiplex IHC is the success of multispectral imaging (MSI).

1.9.2 Multispectral imaging

Analysis of multiplex IHC slides is limited by antigen co-localisation and the spatial
chromogen/fluorophore overlap. The introduction of MSI analysis, makes is possible
to distinguish between co-localised chromogen/fluorophore colours and also
addresses the issue of auto-flourescence.

The functional unit of life is the cell, it performs its functions as a result of
interactions between its diverse components. Understanding the role of each of the
components within the spatial and temporal context of the cell requires the
visualisation of these components. Multiplex based analysis becomes important
within this context. Multiplex IHC benefits from the use of MSI analysis solutions.
Imaging is widely used in geology for remote sensing, this involves the Fourier
transformation of an interferometric image. MSI in biological systems aims to
resolve the constituent spectra in each pixel of the image. The optics underlying this
approach benefit from early descriptions of the path and components of incident
light.

Since Newton’s prism experiments describing the spectrum of visible light there
has been considerable interest in characterising and describing the perception of
colour by the human eye. The human eye is unable to distinguish between closely
related components of the continuous visible spectra (Figure 1.6) for example
different shades/intensities of the colour red. Metamerism occurs when different
spectra are interpreted as the same spectrum by an individual. Presently it is accepted
that object colour perception is dependent on the properties of incident light (transmittance, absorbance and reflection) and intrinsic colour of the surface. For opaque objects the perception of colour is due to the amount of light absorbed and reflected by the object. A problem arises when the light source or its incident angle changes. Bright field microscopy of biological specimens controls for geometry and homogeneity of incident light and so is less susceptible to the phenomenon of reflectance. The perception of colour in biological specimens is largely dependent on its absorbance. The absorbance in these specimens can be related to the amount/concentration of the biological agents (Beer-Lamberts law) and forms the basis of spectrophotometry (Hardeberg et al 2002).

![The Electromagnetic Spectrum](image)

Figure 1.6 The electromagnetic spectrum showing the visible spectrum between 400 to 800nm

The already complex optics operational in bright field microscopy, is further complicated by localising two or more chromogens in close proximity. To surmount the optical challenges, an MSI capturing and resolution system has been developed. MSI works on the principle of differentiation of the visible light spectrum according to its wavelength. The image acquisition system consists of a high speed camera, an adjustable filter, and an illumination source. The camera is usually of a high resolution capable of taking several images in milliseconds. Closely related to the camera is a tuneable filter which splits the image into its constitutive wavelengths. The tunable filter allows for relatively quicker transitions between wavelengths. The interplay between the camera and filter allows for the spectral resolution of the constituent pixels in the image (Levenson and Mansfield 2006). Having a uniform illumination source linked to the equipment allows for stability and reproducibility of the method.
The Nuance™ spectral imaging system consists of a camera mounted onto a conventional microscope equipped with a filter cube operating the liquid crystal tuneable technology. The spectral images taken by the camera are stacked on each other and relate to each individual pixel on the whole image (Figure 1.7). As such it is possible to resolve the spectral properties of every pixel in the image. This system is linked to inForm image analysis software which resolves the pixels into biologically identifiable features (cellular structures, tissue organisation). This system as a whole is then able to analyse multiplex IHC slides. As stated earlier, MSI has important implications for clinical research, but is not without its issues. The pixel based approach of the present solution means that there is a chance of misclassifying biological/non biological phenomenon wrongly.

Figure 1.7 A spectral image cube. Each point represents a pixel and the spectrum of the cube is described by $I(m,n,\lambda)$ where $m$ and $n$ are height and width respectively, and $\lambda$ represents the wavelengths. Adapted from (Garini, 2006).
1.10 Rationale for the study

This study will contribute to the information available in the evolving landscape of oropharyngeal cancer with specific reference to diagnosis and prognosis. Standardised assessment of HPV in OPSCC is important because of: 1) the widespread geographical differences in the reported increases in incidence and prevalence of HPV in OPSCC; 2) the observed differences in prognosis between HPV positive and negative OPSCC; and 3) patient selection in on-going trials of treatment de-escalation (Melchers et al. 2015). As summarised above the popular single detection methods do not properly classify HPV positive and negative OPSCC. Therefore detection algorithms with complementary HPV tests have been suggested (Smeets et al. 2007, Braakhuis et al. 2009, Thavaraj et al. 2011). In their study, Smeets et al reported that a two-step protocol with initial p16 IHC and subsequent GP5+/6+ PCR on positive patients demonstrated 100% sensitivity and specificity when compared to HPV 16 E6 mRNA by RT-PCR (Smeets et al. 2007). Similarly, Thavaraj et al reported that using a consensus three tier staged algorithm (p16 IHC/HPV ISH/HPV PCR) they were able to classify 98% as either HPV positive and negative. These observation demonstrate: 1) the validity of a two-step algorithm is dependent on comparison to the “gold standard” HPV mRNA detection; and 2) a consensus method without comparison to the gold standard will fail to classify all tumours. Therefore an ideal comparison will involve a three tier test to HPV mRNA status. Also, more efficient detection algorithms or preferentially all encompassing newer detection methods are required. New methods should ideally be able to: 1) detect the mRNA of high-risk HPV reported so far in OPSCC (16, 18, 31, 33, 45, 52, 58) (Michaud et al. 2014); 2) should be quantifiable tests not overly dependent on user interpretation; 3) allow for comparison of the HPV mRNA levels to other factors related to prognosis in OPSCC; and 4) the method should have a quick turnaround time. Any test developed will have important implications for HPV detection in OPSCC. This study will describe the development a clinically applicable test for HPV mRNA quantification in FFPE samples using the QuantiGene platform.

In addition, morphological based analysis of biomarkers using IHC is an effective way of defining and characterising the location and expression of target molecules (Webster and Dunstan 2014). Quantification of IHC (instead of current binary classification) will ensure standardisation across centres and its inclusion amongst
reliable assays. In addition, simultaneous identification of multiple markers, while preserving morphological information in the context of quantifiable IHC, extracts more reliable/reproducible information from a single section. This approach is now possible with the improvements in digital pathology (Webster and Dunstan 2014) and spectral unmixing of chromogens and fluorophores (Levenson 2006, Rimm 2006). Chromogens and fluorophores used in a multiplex IHC are limited by bulky substrates and number of possible combinations, respectively (Carvajal-Hausdorf et al. 2015). In their approach, Gerdes et al used sequential staining followed by alkaline quenching to demonstrate up to 61 targets in a single section. Using this approach, linear quantification was limited by decreased detection sensitivity with repeated dye inactivation (Gerdes et al. 2013). Therefore, multiplex IHC staining methods retaining chromogens or fluorophore on stained sections are preferable. However, studies are required to quantitatively validate and to demonstrate comparability to single IHC. In addition, studies need to demonstrate the limits of the multiplex IHC combinations recognising the limitations or chromogen and fluorophore based staining. This study will describe the validation of a chromogenic and fluorescent multiplex IHC using the Vectra MSI platform and inForm automated image analysis solution.

As stated above it is now established that the adaptive immune system plays a role in prevention, development and control of cancers (Galon et al. 2012). Furthermore, available evidence demonstrates that evaluation of immune cell markers in pre-treatment biopsies can be used for prognostic stratification (Gooden et al. 2011). Galon et al described how an immunoscore (CD3/CD45RO, CD3/CD8 or CD8/CD45RO) quantified in the invasive margin or centre of a tumour was superior to the TNM classification in colo-rectal cancer (Galon et al. 2012). Similarly, Angell et al described an “immune contexture” to provide information on the type, functional orientation, density and location of immune cells in the tumour micro-environment as a method for evaluating the prognostic role of immune cells in cancers (Angell and Galon 2013). This approach will standardise TIL studies and possibly reduce the discordant results (as stated above) reported in OPSCC. Quantitative multiplex IHC complemented by automated image analysis can be best applied to evaluate and characterise the local immune micro-environment around tumours. This is due to the complex interactions and network of infiltrating cells not
easily visualised in single stained slides. These studies are needed in well-defined subsets of HPV positive and negative OPSCC in order to evaluate differences in the immune densities in the tumour micro-environment in both groups and their effects on patient outcomes.

Increased infiltration with adaptive immune cells will not be beneficial to most patients due to inter-patient diversity and possibly the role of immune-inhibitory pathways (Murakami and Riella 2014). HPV associated tumours are reported to be more immunogenic than HPV negative tumours (King et al. 2014). It is also reported that the interaction of PD1/PD-L1 creates an immune privileged site allowing for persistence of viral infection and promotion of oncogenesis (Lyford-Pike et al. 2013). Therefore targeting this pathway has important implications in OPSCC. At present several antibodies targeting this (PD1/PD-L1) and ancillary (CTLA-4) pathways are being evaluated in clinical trials (Ji et al. 2012, Moreno and Ribas 2015). However, predictive markers identifying patients likely to benefit from this therapeutic approach are needed. The use of PD1 and/or PD-L1 as predictive markers needs to be evaluated in a prognostic context. In addition, studies evaluating the relationship between expression of the PD1/PD-L1 markers and immune cell density are required to contextualise immune cell infiltration in the presence of immune inhibitory markers. Evaluating the immune characteristics in HPV positive and negative OPSCC is important because of their differing aetiological origins.

Similarly, tumour hypoxia is associated with a poor prognosis in several cancer types (Vaupel and Mayer 2007, Swartz et al. 2015). In HPV associated cervical cancer, hypoxia is associated with an unfavourable prognosis. However, Lassen et al reported a differential response to hypoxia modification with Nimorazole in HPV positive and negative patients, and suggested that tumour hypoxia may not be clinically relevant in HPV associated head and neck cancers (Lassen et al. 2010). However, others have reported similar levels of hypoxia in HPV positive and negative tumours using gene expression profiles (Toustrup et al. 2012), 18F-fluorooazomycin arabinoside (FAZA) positron emission tomography (PET) imaging (Mortensen et al. 2012) and in cell lines (Sorensen et al. 2013). More studies are needed to evaluate whether there is a differential effect of tumour hypoxia in HPV positive patients and negative patients. The work is important because it impacts
whether patients with HPV positive OPSCC should be included or excluded from hypoxia modification trials.

The hypotheses underlying this project were: 1) There will be differences with single HPV detection which would be linked to observable difference in patient survival; 2) multiplex IHC enumeration of TILs will be comparable to single section IHC, in addition multiplex IHC will allow for the evaluation of more antigens on a single slide; 3) there will be a differences in TIL density in HPV positive and negative tumours, infiltration with higher TILs density will be linked to patient survival in both HPV positive and negative patients; 4) there will be differences in PD1/PD-L1 expression in HPV positive and negative tumours which will be linked to patient survival; 5) there is a difference in the hypoxia response in HPV positive and negative patients and high versus low expression of hypoxia markers will be linked to patient survival in both HPV positive and negative patients.

1.11 Aims of the study

The specific aims of the work carried out in this theses were to:

1. Develop a clinical test for HPV detection in OPSCC and compare with current detection methods.

2. Quantitatively validate a multiplex IHC method for TIL enumeration using multispectral imaging.

3. Evaluate the differences in TIL density and localisation in HPV positive and negative OPSCC and observe for the relationship between TIL density and survival

4. Evaluate the PD1-PD-L1 pathway and relationship to T cell density in OPSCC

5. Evaluate the relationships between tumour hypoxia, HPV status and patient survival in OPSCC
2.0 Development of a clinical test for HPV detection in OPSCC and comparison with current detection methods.

2.1 Introduction

Human papillomavirus (HPV) is responsible for over 600,000 cancers worldwide. In recent times its role in head and neck cancers, specifically cancers of tonsil and tongue base sites in the oropharynx has generated considerable interest. Differences in epidemiology and aetiology notwithstanding, there are further contrasts between HPV positive and negative oropharyngeal squamous cell carcinoma (OPSCC). The significant differences in patient characteristics, tumour biology, tumour histology and survival features exhibited by patients with HPV driven OPSCC makes the identification of these patients important for not only proper diagnosis but also selection of alternative treatment approaches with comparatively less associated morbidity.

The biology of HPV infections and resulting cancers has been largely studied in cervical cancer models. It is assumed that the pathological models are similar in OPSCC as both tissues share similar morphological features (Pannone et al. 2011). At present, “popular” methods detect viral nucleic acids, proteins and/or surrogate signs of viral infection. These methods have been useful for studying broad differences between both groups. However, in the context of “individualised therapy” with prognostic biomarkers leading up to treatment choices, inflexible HPV detection is required.

Polymerase chain reaction (PCR), in situ hybridisation (ISH) detection of HPV DNA and p16 immunohistochemistry (IHC) are the most common detection methods in OPSCC. HPV DNA ISH allows for precise spatial localisation and state (integrated versus non-integrated) of the HPV genome in the tissue. It is a method with high specificity, but it suffers from low sensitivity and a longer assay time (Abreu et al. 2012). In addition, HPV DNA ISH requires the presence of a high viral copy number to be detected (Badaracco et al. 2000). Polymerase chain reaction (PCR) DNA based methods avoid the problem of low viral copy number seen with ISH. The method is sensitive, specific and can be used to detect different HPV types. However, using the consensus primers approach which target conserved regions on the HPV genome (mostly the L1) there is the risk of: 1) false negatives due to L1 loss, and 2) competition for reagents by other less common HPV types allowing for
underestimation of HPV co-infection (Brandstetter et al. 2010). Furthermore, the presence of HPV DNA as detected by PCR may be a passenger infection rather than due to cancer. p16 is a cyclin-dependent kinase inhibitor that is up-regulated when released by HPV E7 suppression of pRB (Li et al. 1994). This observation allows p16 overexpression assessed using IHC to be used as surrogate marker for HPV positive cancers (Singhi and Westra 2010). However, p16 overexpression is not exclusively related to HPV driven oncogenesis. Mutations of CDK2NA encoding p16 (p.Arg112dup) are found in several other cancer types (melanoma, pancreas, respiratory, gynaecological, head and neck, breast, urinary, CNS) and this has been linked to cigarette smoking (Helgadottir et al. 2014) another aetiological factor in OPSCC. Available evidence thus far suggests that p16 although sensitive is highly non-specific for HPV detection.

As stated above, there are issues with the current detection paradigms which result in downstream effects on treatment and/or biomarker development approaches dependent on exacting HPV detection. Several algorithms have been suggested for HPV detection in OPSCC (Smeets et al. 2007, Braakhuis et al. 2009, Thavaraj et al. 2011). An ideal HPV diagnostic method will aim to detect and directly quantify transcriptionally active E6 and E7 mRNA of the high risk HPV types and possibly relate this to other prognostic markers operational in OPSCC. Such multiplexing is becoming important in clinical and research where more information is required from small amounts of tissue.

In this study, a quantitative HPV detection test was developed which allows for 1) HPV typing, 2) identification of transcriptionally active HPV, and 3) the detection of other prognostic biomarkers in OPSCC. The aim was to promote the adaptation of a different and more robust detection paradigm in HPV positive OPSCC. The method described could be modified to accommodate inclusion of other prognostic biomarkers.
2.2 Materials and methods

2.2.1 Patients

A retrospective audit using a radiotherapy database at The Christie NHS Foundation Trust Hospital identified patients with a confirmed histological diagnosis of OPSCC. Patients were treated between January 2002 – December 2011 and radiotherapy was one or the only therapy modality. Four treatment options were available to patients in this study: radiotherapy alone, radiotherapy in combination with chemotherapy and/or surgery. 50% of the patients received radiotherapy alone, 22% received radiotherapy post surgery, 17% received radiotherapy in combination with chemotherapy and 11% had surgery in combination with chemoradiotherapy. Patient clinico-pathologic and outcome data were collected from the case notes and The Christie Head and Neck assessment forms. Pre-treatment formalin-fixed paraffin-embedded (FFPE) blocks obtained at pre-treatment biopsy were then requested. Ethical approval for the study was obtained from the local research ethics committee (03/TG/076).

2.2.2 HPV detection

1) \textit{p16 expression}: Tumour sections were stained with haematoxylin and eosin to confirm tumour presence prior to assessing HPV status. Detection of p16 used the CINtec histology kit (Roche, Basel, Switzerland) and a Biogenix i6000 autostainer (Biogenix, Fremont CA, USA). HPV positive and negative controls were included in each staining batch. Example staining is shown in Figure 2.1. Tumours were scored as positive if there was a strong and diffuse brown staining of the nucleus and cytoplasm in $\geq 70\%$ of the tumour specimen as previously described and validated by others (Ang et al. 2010, Singhi and Westra 2010, Chung et al. 2014). The slides were scored twice, by a single scorer with 91% concordance.

2) \textit{In Situ hybridization}: The ISH assay (Ventana Medical Systems, Arizona, USA) was performed at Manchester Royal Infirmary according to the manufacturer's guidelines using the BenchMark automated slide staining system. The Inform HPV III probe sets were able to detect oncogenic HPV 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, and 70. HPV DNA ISH slides were scored by two independent observers (80% concordance) and any discrepancies resolved by re-evaluation by a pathologist.
A positive score was awarded only for punctate, blue-coloured staining within the nuclei of tumour cells. Diffuse staining of the nuclei was scored as negative result.

3) **HPV DNA PCR**: HPV DNA-positive samples (by SPF10 DEIA) were genotyped using the INNO-LiPA HPV genotyping assays and the Roche Linear Array HPV genotyping test performed at the Institute of Cancer and Genetics, School of Medicine at Cardiff University according to protocol. Tissue was strictly processed with the appropriate RNA procedures and samples where controls failed to amplify were excluded.

**2.2.3 HPV mRNA sequence selection**

A literature search was conducted to identify the common HPV types in OPSCC (Chaturvedi et al. 2011, Kreimer and Chaturvedi 2011). Seven HR-HPV types (16, 18, 33,35,45,52 and 58) were identified. The GenBank accession number of reference and variants for each of the HR-HPV types were obtained (Burk et al. 2013). E2, E6 and E7 sequences of the reference (RefSeq) and its variants were obtained from the National Centre for Biotechnical Information (NCBI) database. Sequence homology was compared using the Basic Local Alignment Search Tool (BLAST) on the NCBI platform to ensure sequence homology amongst variants to the reference sequence (Table 2.1). The RefSeq of E2, E6 and E7 of the HPV types above, RB, TP53, CDKNA2 (P16) and five housekeeping genes (B2M, RPL11, GNB2L1, HPRT1, and RPL24) were compared and the least homologous regions of the respective sequence used to construct a multiplex assay.
Figure 2.1 Examples of p16 staining:
(A) p16 positive tumour (W034) as evidenced by widespread diffuse brown staining of the cytoplasm and nucleus; (B) negative control (W034); (C) p16 negative tumour (W036) as evidenced by absence of the diffuse brown staining; (D) negative control (W036).
Figure 2.2 Examples of HPV DNA ISH staining:
(A) HPV DNA ISH positive showing punctate staining characteristic of integrated HPV DNA
(B) HPV DNA ISH showing diffuse staining characteristic of episomal HPV DNA (C) HPV DNA ISH negative section and (D) Negative control.
Table 2.1 Percentage homology of designed probesets compared to Affymetrix library sequence and reference sequence for the HPV

<table>
<thead>
<tr>
<th>Designed probeset</th>
<th>Affymetrix sequence (%)</th>
<th>HPV ref sequence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV16_E2</td>
<td>100</td>
<td>99.83</td>
</tr>
<tr>
<td>HPV16_E6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HPV16_E7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HPV18_E2</td>
<td>100</td>
<td>99.79</td>
</tr>
<tr>
<td>HPV18_E6</td>
<td>100</td>
<td>98.46</td>
</tr>
<tr>
<td>HPV18_E7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HPV33_E2</td>
<td>100</td>
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</tr>
<tr>
<td>HPV33_E6</td>
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</tr>
<tr>
<td>HPV33_E7</td>
<td>100</td>
<td>99.62</td>
</tr>
<tr>
<td>HPV35_E2</td>
<td>96.59</td>
<td>100</td>
</tr>
<tr>
<td>HPV35_E6</td>
<td>98.82</td>
<td>99.05</td>
</tr>
<tr>
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<td>99.29</td>
<td>99.65</td>
</tr>
<tr>
<td>HPV45_E2</td>
<td>100</td>
<td>99.47</td>
</tr>
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<td>99.25</td>
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</tr>
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<td>HPV52_E2</td>
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<td>99.53</td>
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<td>HPV52_E7</td>
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<td>HPV58_E2</td>
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<td>99.75</td>
</tr>
<tr>
<td>HPV58_E7</td>
<td>100</td>
<td>98.42</td>
</tr>
</tbody>
</table>

2.2.4 QuantiGene detection of HPV genes

The tissue sections were diluted with proteinase K in homogenizing solution and incubated at 65°C for 6 hours. A working solution of lysis mixture, blocking reagent, proteinase K, magnetic capture beads and nuclease free water was introduced to the tissue homogenate in their respective wells on a magnetic separation plate. The plate was pressure sealed and incubated for 20 hours at 55°C. Pre-amplifier, amplifier, label and Streptavidin Phycoerythrin (SAPE) were applied in series to the samples all interspersed by a washing steps. Subsequently, the plate and the beads were diluted using SAPE buffer and read using the Bio-Plex luminex reader.
2.2.5 Data analysis

The results from the plate reader were exported to Microsoft Excel for data handling and subsequently compared and charts generated using SPSS version 20.0 and GraphPad Prism. For the box plots, Shapiro-Wilk normality test was used to test for the distribution of the data. As the data were not normally distributed, the Mann-Whitney test for comparing the medians was used. Actuarial calculations of overall survival were obtained using the Kaplan-Meier method. Univariate analysis was made using the Log Rank (Mantel-Cox) method. Chi-squared test was used to compare categorical data and the threshold for statistical significance was set at 0.05.

2.3 Results

2.3.1 Patient characteristics

Ninety patients with p16, ISH DNA and HPV DNA PCR data were included in this study. There were 10 sample failures with the quantigene method, data from these patients were excluded from further analysis. The data from this did not contribute to either positive or negative HPV classification. The number of positive and negative patients changed between tests. The positivity rates were 57% (p16), 55% (PCR), 50% (ISH) and 52% (QuantiGene). Differences were also observed in the patient characteristics which changed between the tests (Table 2.2).

Comparing the percentage difference in the patient characteristics between HPV positive and HPV negative in the three test showed no significant differences according to gender and alcohol use. However, smoking history did differ significantly in the HPV positive vs. negative OPSCC as classified by HPV DNA PCR (P=0.05) and Quantigene mRNA (P<0.0001) detection. AJCC prognostic grouping differed only by Quantigene mRNA classification (P=0.001). Tumour grade was significantly different as classified by HPV DNA PCR (P=0.01) and ISH (P=0.04). There were more non-smokers and high stage tumours in HPV negative patients detected by the QuantiGene methods (Table 2.2).
Table 2. 2 Patient characteristics by the different HPV detection methods

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>P16 N (%)</th>
<th>PCR N (%)</th>
<th>ISH N (%)</th>
<th>QuantiGene N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos</td>
<td>Neg</td>
<td>P</td>
<td>Pos</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>38 (83)</td>
<td>25 (74)</td>
<td>0.16</td>
<td>34 (77)</td>
</tr>
<tr>
<td>Female</td>
<td>8 (17)</td>
<td>9 (26)</td>
<td></td>
<td>10 (23)</td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>8 (17)</td>
<td>4 (12)</td>
<td>0.45</td>
<td>5 (13)</td>
</tr>
<tr>
<td>Some</td>
<td>34 (74)</td>
<td>27 (79)</td>
<td></td>
<td>35 (78)</td>
</tr>
<tr>
<td>Heavy</td>
<td>4 (9)</td>
<td>3 (9)</td>
<td></td>
<td>4 (9)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>10 (22)</td>
<td>6 (18)</td>
<td>0.36</td>
<td>11 (25)</td>
</tr>
<tr>
<td>Ex</td>
<td>20 (43)</td>
<td>18 (53)</td>
<td></td>
<td>18 (39)</td>
</tr>
<tr>
<td>Current</td>
<td>16 (35)</td>
<td>10 (29)</td>
<td></td>
<td>16 (36)</td>
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</tr>
<tr>
<td>1</td>
<td>1 (2)</td>
<td>2 (6)</td>
<td></td>
<td>1 (2)</td>
</tr>
<tr>
<td>2</td>
<td>4 (9)</td>
<td>3 (9)</td>
<td></td>
<td>4 (9)</td>
</tr>
<tr>
<td>3</td>
<td>9 (20)</td>
<td>6 (17)</td>
<td></td>
<td>9 (21)</td>
</tr>
<tr>
<td>4</td>
<td>32 (69)</td>
<td>23 (68)</td>
<td></td>
<td>30 (68)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>2 (4)</td>
<td>1 (3)</td>
<td>0.13</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Mod</td>
<td>22 (48)</td>
<td>21 (62)</td>
<td></td>
<td>22 (50)</td>
</tr>
<tr>
<td>poorly</td>
<td>22 (48)</td>
<td>12 (35)</td>
<td></td>
<td>21 (48)</td>
</tr>
</tbody>
</table>

PCR – polymerase chain reaction, ISH – in-situ hybridisation, Pos- positive, Neg – negative, well – well differentiated, Mod – moderately differentiated, poorly – poorly differentiated, P –values for pos and neg compares the positive and negative columns respectively were generated using Chi-squared test.
2.3.2 HPV detection method comparison

Table 2.3 summarises the data comparing the different HPV detection methods (p16 IHC, HPV DNA PCR and HPV DNA ISH) to QuantiGene HPV mRNA detection in terms of sensitivity and specificity. The sensitivity of the different tests were as follows: p16 IHC (85%), ISH (78%), PCR (80%) and combination of the three test (85%).

The ROC curve (Figure 2.2) shows the area under the curve for the comparison with the QuantiGene mRNA method. Under non-parametric statistics the tests were all significantly related to the comparator test (QuantiGene). The QuantiGene assay also identified a tumour positive for HPV 18 and another for HPV 33 which were negative with other HPV detection tests. The rest were positive for HPV 16 by the QuantiGene method.

Table 2.3 Comparison of the different HPV detection methods

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Area under the curve (AUC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>85</td>
<td>77</td>
<td>69</td>
<td>75</td>
<td>0.81*</td>
</tr>
<tr>
<td>ISH</td>
<td>78</td>
<td>87</td>
<td>86</td>
<td>80</td>
<td>0.82*</td>
</tr>
<tr>
<td>PCR</td>
<td>80</td>
<td>69</td>
<td>73</td>
<td>71</td>
<td>0.74*</td>
</tr>
<tr>
<td>3 tests</td>
<td>85</td>
<td>88</td>
<td>97</td>
<td>80</td>
<td>0.88*</td>
</tr>
</tbody>
</table>

*P-values comparing the tests to QuantiGene mRNA detection, all P values were <0.0001
2.3.3 Housekeeping genes

The log 2 of the mRNA expression levels of the five housekeeping genes were obtained. The distribution of the data was evaluated using the Q-Q plots (expected versus observed plots) and the Shapiro-Wilk normality tests. Only two housekeeping genes (HPRT1 and RPL24) passed the test for normality. Furthermore, the correlation plots of the log 2 of four housekeeping genes compared to one (B2M) showed highly significant strong correlations for three of the housekeeping genes (RPL11, GNB2L1, RPL24). HPRT1 had a weak relationship with the other housekeeping genes (Figure 2.3). Similarly, a matrix of Spearman correlations of the housekeeping genes showed highly significant (P<0.0001) correlations between four of the housekeeping genes (B2M, RPL11, GNB2L1 and RPL24); Table 2.4. A two factor principal component analysis using an oblique rotated solution showed a
strong relationship between the four housekeeping genes \((B2M, RPL11, GNB2L1\) and \(RPL24\)) in the first component with a low/negative relationship in the second component (Table 2.5). This three level analysis allowed for the exclusion of the \(HPRT1\) from further analyses.

Figure 2.3 Correlation plots in log 2 of the five housekeeping genes. Correlations shown in relation to \(B2M\). \(RPL11\), \(GNB2L1\), \(RPL24\) showed high \(r^2\) values (0.88, 0.82 and 0.80 respectively). \(HPRT1\) had a low \(r^2\) value of 0.15.

Table 2.4 Spearman correlation matrix of the housekeeping genes

<table>
<thead>
<tr>
<th></th>
<th>B2M</th>
<th>RPL11</th>
<th>GNB2L1</th>
<th>HPRT1</th>
<th>RPL24</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M</td>
<td>0.92</td>
<td>0.92</td>
<td>0.43</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>RPL11</td>
<td>0.92</td>
<td>0.94</td>
<td>0.36</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>GNB2L1</td>
<td>0.92</td>
<td>0.94</td>
<td>0.36</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>HPRT1</td>
<td>0.43</td>
<td>0.36</td>
<td>0.36</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>RPL24</td>
<td>0.90</td>
<td>0.90</td>
<td>0.88</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.5 Pattern matrix of the principal component analysis for the housekeeping genes

<table>
<thead>
<tr>
<th>Housekeeping genes</th>
<th>Component 1</th>
<th>Component 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL11</td>
<td>1.00</td>
<td>-0.058</td>
</tr>
<tr>
<td>GNB2L1</td>
<td>0.97</td>
<td>-0.034</td>
</tr>
<tr>
<td>B2M</td>
<td>0.95</td>
<td>0.029</td>
</tr>
<tr>
<td>RPL24</td>
<td>0.92</td>
<td>0.078</td>
</tr>
<tr>
<td>HPRT1</td>
<td>0.004</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Rotation Method: Oblimin with Kaiser Normalization. Rotation converged in 3 iterations

2.3.4 QuantiGene mRNA

The geometric mean of the mRNA expression of the four selected housekeeping genes was used to normalise the mRNA profiles in the QuantiGene plex experiment. A Spearman correlation matrix of the normalised plex set showed a highly significant positive correlation (P<0.0001) between the HPV genes. Also observed was a significant positive correlations between the expression of CDKN2A (p16) and the HPV genes E2 (P=0.004), E6 (P=0.004) and E7 (P=0.003). There were no correlations between expression of the HPV genes and expression of TP53 and RB (Table 2.6).

Table 2.6 Spearman correlation matrix of the RNA expression of genes in the QuantiGene plex set

<table>
<thead>
<tr>
<th></th>
<th>E2</th>
<th>E6</th>
<th>E7</th>
<th>TP63</th>
<th>TP53</th>
<th>RB</th>
<th>CDK</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>0.88</td>
<td>0.83</td>
<td>-0.18</td>
<td>-0.07</td>
<td>-0.12</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>0.88</td>
<td>0.94</td>
<td>-0.17</td>
<td>-0.09</td>
<td>-0.25</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>E7</td>
<td>0.83</td>
<td>0.94</td>
<td>-0.15</td>
<td>-0.05</td>
<td>-0.23</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>P63</td>
<td>-0.18</td>
<td>-0.18</td>
<td>-0.15</td>
<td>0.38</td>
<td>-0.08</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>-0.07</td>
<td>-0.09</td>
<td>-0.05</td>
<td>-0.38</td>
<td>-0.35</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>RB</td>
<td>-0.12</td>
<td>-0.25</td>
<td>-0.23</td>
<td>-0.07</td>
<td>-0.35</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>CDKN2A</td>
<td>0.33</td>
<td>0.32</td>
<td>0.33</td>
<td>0.19</td>
<td>0.11</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

Not significant
2.3.5 Survival analysis

Table 2.7 summarises the results of the univariate analysis of the 80 patients in this study. There were no significant differences in OS or LRC when stratified by HPV DNA ISH. Stratifying by HPV DNA PCR and p16 IHC status showed a significant difference in OS (P=0.02 and P=0.04) but not LRC (P=0.62 and 0.39). However, stratifying by HPV mRNA status showed significant differences in both OS (P=0.005) and LRC (P=0.02).

Figure 2.4 shows sub stratification by mRNA positive and negative patients and each of the three HPV detection methods (p16, HPV DNA ISH and HPV DNA PCR) status to obtain four groups (as shown in figure 2.4). p16 positive and negative patients in the different arms of mRNA stratification showed a non-significant (P=0.16) and a significant (P=0.01) difference for p16 negative and positive patients respectively. Similar analysis on the HPV DNA ISH stratified patients showed a non-significant difference and significant difference in the negative (P=0.19) and positive (P=0.0007) groups respectively. Replicating the analysis on the PCR negative and positive patients showed a non-significant (P=0.90) and significant (P=0.02) difference respectively. Using the three test to sub stratify mRNA positive and negative showed no significant difference for both negative (P=0.82) and positive (P=0.38) patients.

Table 2.7 Summary of the univariate analysis

<table>
<thead>
<tr>
<th>Detection methods</th>
<th>Status</th>
<th>Overall survival</th>
<th>Loco-regional control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean survival</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>p16 IHC</td>
<td>Positive</td>
<td>45 (months)</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>Positive</td>
<td>51 (months)</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>ISH</td>
<td>Positive</td>
<td>47 (months)</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>All (3 tests)</td>
<td>Positive</td>
<td>51 (months)</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>HPV mRNA</td>
<td>Positive</td>
<td>52 (months)</td>
<td>4.26</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.4 Kaplan-Meier plots of overall survival. (A) mRNA and/or p16 positive and negative patients, (B) mRNA and/or ISH positive and negative patients, (C) mRNA and/or PCR positive and negative and (D) mRNA vs. all positive/negative and or discordant patients. Sub stratification of p16 or ISH positive and negative patients using mRNA showed significant differences only in the positive patients (P=0.01, P=0.0007). PCR sub stratification (using mRNA detection) of positive and negative patients showed only significant differences in the negative patients (P=0.02). Sub stratification of concordant positive/negative patients showed no significant difference between positive and negative patients respectively (P=0.82, P=0.38).
2.4 Discussion

The data in this study demonstrates that HPV detection and/or patient stratification for prognosis in OPSCC should preferably be done using a combination of tests or E6/E7 mRNA detection. The method of multiplex mRNA detection described in this study presents the best opportunity for HPV mRNA detection/typing and also allows for the identification of other prognostic factors operational in HPV positive and/or negative patients. Discrepancies in HPV testing results between methods are under reported in cervical cancer types, because few are HPV negative. Conversely with the dichotomy of HPV status and its relevance in OPSCC, accurate HPV detection is required. Detection of HPV mRNA in cancers allows for the identification of transcriptionally active HPV (Ukpo et al. 2011, Schache et al. 2013). In addition, others have described increasing HPV mRNA levels with increasing severity of disease (Sotlar et al. 2004, Lie et al. 2005, Castle et al. 2007). The use of the method described in this study will allow for accurate HPV detection, typing and quantification in OPSCC.

In this study, a mRNA plex set which detects the common HPV types identified in OPSCC so far was designed. This approach allowed for not only HPV transcriptional activity detection but also HPV typing. A systematic review by Kreimer et al reported that HPV types 16 and 18 were responsible for 97% and 3% respectively of HPV positive OPSCC with other types playing lesser roles (Kreimer et al. 2005). It is pertinent to note that in their review the authors included only studies using PCR for HPV typing which may have led to an overestimation of the true prevalence of different HPV types. Using a single, possibly imprecise method for typing and potentially describing the patient population could further limit biomarker development. However, HPV 16 does play a predominant role in OPSCC.

In this study, some patient characteristics differed between the different tests. Smoking status has been suggested as an additional prognostic factor in HPV positive OPSCC (Ang et al. 2010, Granata et al. 2012, Tinhofer et al. 2015) with proper identification of HPV positive smokers necessary to test any association. This study found a significantly higher percentage of smokers were HPV negative (by Quantigene mRNA) compared to p16, HPV DNA PCR/ISH. The imprecise HPV detection could potentially explain the observation by others of poor survival in the
HPV positive smokers (Ang et al. 2010). Re-analysis with more comprehensive HPV detection methods might produce a different observations.

In this study comparing the tests to the “gold standard” HPV mRNA detection (Gillison and Shah 2003) showed that amongst the single tests (p16 IHC, HPV DNA PCR/ISH) p16 IHC and HPV DNA ISH have the highest sensitivity and specificity respectively when compared to QuantiGene HPV detection. Using the combination of the three tests to stratify patients increased sensitivity and specificity. Comparing the AUC of the single tests showed that HPV DNA ISH had the highest value. Discrepancies amongst common HPV detection methods in head and neck cancer are well reported. In a study comparing four approaches (p16 IHC, HPV DNA ISH, HR-HPV DNA and MY-PCR) to HPV E6/E7 RNA expression as measured by RT-PCR, Schlecht et al reported that p16 IHC performed better than ISH in both comparisons (Schlecht et al. 2011). It is recognised that the use of different p16 antibodies affects sensitivity and specificity profiles (Agoston et al. 2010, Singhi and Westra 2010, Schlecht et al. 2011). Furthermore, HPV DNA ISH scoring can be challenging as a result of the differing pattern of hybridisation and signal which is dependent on the localisation/activity of the virus (Unger 2000).

Methodological differences apart, p16 is at best a surrogate marker for HPV oncogenesis. P16 positive tumours could be aetiologically linked to HPV or not (Jordan et al. 2012, Cancer Genome Atlas Research et al. 2013, Lingen et al. 2013, Zuo et al. 2013). Expression of p16 is assumed to be universal in HPV positive tumours with discrepancies linked to methodological and/or scoring differences (Seiwert et al. 2015). However, mutations in RB and CDKN2A pathways have been reported, and if present will produce phenotypes similar to HPV negative tumours (Cancer Genome Atlas Research et al. 2013, Rietbergen et al. 2014). Therefore the use of p16 IHC as a stand-alone test for treatment decisions is suspect. In our study, p16 IHC although significantly associated with HPV mRNA detection underperformed when compared to others as a stand-alone test. In this study the use of a more specific test as HPV DNA ISH test as a confirmatory is advisable particularly in a double step algorithm.

Following normalisation to housekeeping genes the relationship was evaluated between the different HPV genes included in the plex. There was a positive correlation between HPV E2, E6 and E7 mRNA expression with the highest
correlation seen between $E6$ and $E7$ mRNA. Also observed was a weak but positive correlation with $CDKN2A$ and a negative correlation with the $RB$ mRNA. In a genome-wide analysis of HPV integration in human cancers, Akagi et al reported recurrent disruption of the $E2$ and other regions of the genome (Akagi et al. 2014). In their observations the disruption of $E2$ is not always linked to reduction in copy numbers of viral sequences. This supports our observation of persistence of $E2$ mRNA despite up-regulation of $E6$ and $E7$ in these patients. Furthermore persistence of HPV in episomes is seen in HPV OPSCC (Miller et al. 2012), and others have described differential methylation of $E2$ binding site 1 and subsequent hyperactivity of the upstream regulatory region leading to oncogenesis (Vinokurova and von Knebel Doeberitz 2011). These factors could also account for the positive correlation of $E2$, $E6$ and $E7$ seen in this study. The positive and negative correlation of the expression of HPV genes with that of $CDKN2A$ and $RB$ is as expected (Yang et al. 2005).

The importance of the differing detection tests is evident when evaluating patient survival. This study found only HPV mRNA, HPV DNA and the combination of the three tests showed a significant relationship with patient survival. Others have reported similar divergence in patient survival dependent on HPV detection methods (Seiwert et al. 2015). Furthermore in this study a significant difference was observed when negative patients as identified by p16 IHC and HPV DNA ISH were further stratified by HPV mRNA status. A similar effect was not seen when negative patients as identified by HPV DNA PCR or the combination of tests were stratified using the HPV mRNA detection method. This suggests there is potential for patient misclassification when using single detection methods which has implications for determining prognosis and selection for treatment de-intensification. In addition it is pertinent to mention that there was a failure rate of 9% with the quantigene method. In comparison to other single HPV detection method it is not known if similar failure rates accounts for the observable differences. Notwithstanding the failure rates are high and should be further investigated in future studies.

In conclusion strongly suggested that issues with HPV testing discrepancies are resolved by multiple testing and/or HPV mRNA quantification in the context of any proposed change to treatment models. It is recommended that a combination of three tests provides the best option for HPV stratification in OPSCC, as this is comparable to HPV mRNA detection.
3.0 Quantitative validation of a multiplex immunohistochemistry method for TIL enumeration using multispectral imaging.

3.1 Introduction

The immunological content of a tumour can affect patient outcome with tumour infiltration by effector T cells associated with a good prognosis in most cancer types (Gooden et al. 2011). In some cases, an immune score was more predictive of outcome than a standard staging system (Galon et al. 2006). Recent studies revealed that characterisation of an immune score in tumours may need to go beyond single antigen staining to increase prognostic value and facilitate inclusion in the routine histopathological assessment of tumours (Goc et al. 2014). Moreover, increased understanding of the inflammatory environment in tumours may yield insight into novel therapeutic approaches.

Identification and quantification of tumour infiltrating lymphocyte (TIL) subpopulations in situ is challenging. Multiparameter flow cytometry can be used but this technique disrupts the tissue and limits interpretation. Immunohistochemistry (IHC) is preferred for contextualisation of TILs in sections (van der Loos 2008). The substantial heterogeneity in the number and spatial distribution of TILs both between and within tumour types limits the use of single-plex IHC. Characterising the heterogeneity in the tumour immune microenvironment relies on identification of phenotypically distinct immune cell sub-populations (e.g. of dendritic cells, macrophages, natural killer cells, T and B lymphocytes).

There is currently no standardisation of TIL evaluation methods, with most studies using manual counting following IHC in successive single-antigen stained formalin-fixed paraffin-embeded (FFPE) tumour sections. The limitations of this process are acknowledged; it is time-consuming, highly subjective and associated with intra- and inter-observer variability (Levenson and Mansfield 2006). The approach limits the potential for comparisons between studies, and there is a need for the development of methods that can improve reproducibility and standardisation across centres.

Multiplex detection of antigens in biological samples provides more information, and helps conserve limited clinical material. In tissue based IHC, multiplex analysis enables the observation of spatial contextual relationships of different antigens. It
also provides an opportunity to limit the effect of heterogeneity associated with comparing successive tumour sections (Stack et al. 2014). The use of chromogenic/fluorescent markers for multiplex IHC was hampered in the past because of problems with the spectral resolution of co-localised chromogens/fluorophores. Recent technical advances have enabled the resurgence of multiplex IHC. First, sequential staining with an intervening unbound antibody removal step was shown to reduce cross-reactivity of antibodies from the same species (van der Loos 2008). Second, covalent interactions of antibodies with chromogens and fluorophores using the tyramide signal amplification [TSA\textsuperscript{TM}] system was shown to allow for repeated IHC staining (Bobrow et al. 1991). Third, antibody titration was shown to minimise the likelihood of molecular shielding of subsequent proximal or co-localised antigens (van der Loos 2008). Fourth, is the development of multispectral imaging (MSI).

Several platforms for MSI are available. The principle involves the use of a camera and spectroscopy to resolve the spatial and spectral properties of chromogens/fluorophores in an image (van der Loos 2008). The optical spectra are resolved for individual pixels within an image cube generated by reflection/emission spectra of different wavelengths (Levenson and Mansfield 2006). The spectrally resolved images can be quantified by image analysis, which not only permits multiplex IHC but also elevates IHC to a method with a quantitative output.

There is a need to validate MSI cell based quantification of multiplexed tumour sections by investigating chromogen/fluorophore loss and possible tissue degradation following repeated staining steps. Any new IHC method must also be shown to be comparable to and/or better than the currently used single marker IHC. This study reports for the first time the validation of a multiplex IHC method for cell based quantification of chromogenic and fluorescent markers using automated MSI analysis solution. The work focuses on the in situ enumeration of TILs, but could be used for studying other antigen panels.
3.2 Materials and methods

3.2.0 Tissue selection

For the chromogen validation experiments, a TMA containing 82 cores constructed from 40 archived pre-diagnostic FFPE biopsies of follicular lymphoma was obtained with consent from the Lymphoma Translational Research Group of the University of Manchester (FLTMA4). For the fluorophore validation experiments, a TMA (QC_KILL_01, 13_RIBY_2) containing 40 cores from 20 archived pre-prepared cancer-wide FFPE sections was used. Both TMA blocks were obtained from the Manchester Cancer Research Centre Biobank. Ethical permission for this study was obtained from the Central Manchester Multi-Centre Research Ethical Committee (03/8/016). The samples were provided by Dr. Richard Byers of the Translational Lymphoma research group. The use of lymphoma samples were to use a specimen enriched with lymphocyte population to allow for adequate comparism.

3.2.1 Multiplex Immunohistochemistry

3.2.1.1 Chromogenic staining:

Figure 2.1 summarises the method used. Monoclonal mouse antibodies directed at CD3, CD8 and FOXP3 positive lymphocytes were selected based on previous experience (Wansom et al. 2012, Nordfors et al. 2013, Balermpas et al. 2014, Ward et al. 2014). The first step in the TMA staining was deparaffinisation of 3 µm sections in xylene and rehydration in graded concentrations of alcohol. Alkaline epitope retrieval was carried out in a pressure cooker at 125°C for 1 min then 90°C for 10 s at a maximum pressure of 20 psi. The slides were then stained in a Biogenix i6000 autostainer (Biogenex, Fremont, CA, USA) and endogenous peroxide blocked using peroxidase solution (ACROS Organics, Geel, Belgium). Table 3.1 summarises the secondary antibodies and visualisation chromogens used for the different antigens. For multiplex IHC, high pH (pH 8.5) heat induced epitope retrieval was carried out between individual marker staining by transferring slides to an antigen retrieval solution and heating for 10 min. The single/multiplex stained slides were then counterstained in Gill’s haematoxylin, rehydrated in graded concentrations of alcohol and cover slipped (Leica CV5030, Leica Biosystem (Wetzlar, Germany)).
3.2.1.2 Fluorescent staining:
Monoclonal mouse antibodies were obtained directed at CD3, CD8, CD4, CD56, CD20 and FOXP3 (Table 3.1, Figure 3.2). The staining was carried out using a Ventana medical systems autostainer (Ventana, Arizona, US). Deparafinisation, high pH (pH 8.5) antigen retrieval and endogenous protein block were applied sequentially. Primary antibody was applied followed by secondary and the corresponding fluorophores (detailed in Table 3.1). For the multiplex fluorescent staining, each antibody-fluorophore step was interspersed by a low pH (pH 6.0) retrieval step. Slides were then coverslipped with an aqueous mount.

![Figure 3.1 Experimental design for validating multiplex chromogen staining.](image)

Four sections from a lymphoma tissue microarray, 3 μm in thickness, were cut serially and labelled according to the order in which they were cut (e.g. section 1, section 2). 3 μm thick sections 1, 3 and 4 were each stained for the separate biomarkers CD3 (Vector Red), CD8 (Vina Green) and FOXP3 (DAB) respectively. Section 2 was labelled for all biomarkers using a multiplex protocol. All conditions and reagent exposures were kept consistent for all sections; primary antibodies were omitted and replaced with isotope controls in the single stained sections.
Figure 3.2 Experimental design for validating multiplex fluorescence staining. Sections from pre-prepared cancer-wide tissue microarray, 3 μm in thickness, were cut serially and labelled according to the order in which they were cut (e.g. section 1, section 2). 3 μm thick sections were stained for separate biomarkers CD3 (Fluorescein), CD4 (Cyanine 5.5), CD8 (cyanine 3), CD20 (cyanine 5) and FOXP3 (Cyanine 3.5) respectively.1,3,5,7, and 9 section were stained using multiplex labelling while 2,4,6,8 and 10 were stained with respective single markers. All conditions and reagent exposures were kept consistent for all sections; primary antibodies were omitted and replaced with isotope controls in the single stained sections.

Table 3.1 Antigen staining and visualisation protocol

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Mouse monoclonal antibody</th>
<th>Secondary antibody</th>
<th>Visualisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>anti-human CD3, clone F7.2.38, 1:50</td>
<td>ImmPRESS™&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Vector red&lt;sup&gt;a&lt;/sup&gt; and Fluorescein&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD8</td>
<td>Mouse monoclonal anti-human, clone C8/144B CD8&lt;sup&gt;d&lt;/sup&gt; 1:50</td>
<td>EnVision®&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Vina green&lt;sup&gt;c&lt;/sup&gt; and Cyanine 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FoxP3</td>
<td>ab20034&lt;sup&gt;e&lt;/sup&gt; 1:40</td>
<td>EnVision®&lt;sup&gt;d&lt;/sup&gt;</td>
<td>DAB&lt;sup&gt;d&lt;/sup&gt; and Cyanine 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4</td>
<td>Monoclonal mouse anti-human, clone 4B12. CD4&lt;sup&gt;d&lt;/sup&gt; 1:50</td>
<td>EnVision®&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cyanine 5.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD20</td>
<td>Monoclonal mouse anti-human, clone L26. CD20&lt;sup&gt;d&lt;/sup&gt; 1:50</td>
<td>EnVision®&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cyanine 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>- Vector Laboratories Inc, Burlingame, CA, USA, <sup>b</sup>- PerkinElmer, Massachusetts, US, <sup>c</sup>- Biocare Medical, CA, USA, <sup>d</sup>- DakoCytomation, Denmark and <sup>e</sup>- Abcam, Cambridge, UK
3.2.2 Multispectral image analysis/scoring

Single and multiple marker stained slides were loaded onto the Vectra automated MSI system (PerkinElmer, Massachusetts, USA). Single stained slides were used to generate spectral libraries that allowed distinction of CD3, CD8, CD20, CD56, CD4 and FoxP3 expression in the multiple marker stained slides for both chromogen and fluorophore stained sections. The spectral libraries and the scanned single/multiplex images were analysed using inForm advanced image analysis software (PerkinElmer, Massachusetts, USA). Cell identification was achieved using a counterstain (haematoxylin/DAPI), which stains all the nuclei present. Following cell identification the single/multiplex marker expression of individual cells were quantified by un-mixing the stains using the pre-generated spectral libraries (Figure 3.3).
Figure 3.3 Preparation of multispectral image cubes in InForm and un-mixing of the MSI into images of component stains:
(A) haematoxylin counterstain of the image; (B) unmixed vina green staining of CD3; (C) unmixed vector red staining of CD8; (D) unmixed DAB staining of FoxP3; and (E) final multiplex image with all four stains. Also shown is the unmixing of the fluorescent images: (F) unmixed DAPI stained cells; (G) DAPI+ cyanine 3 (CD8+); (H) DAPI + cyanine 3 (CD8+) + cyanine 3.5 (FoxP3+); (I) DAPI + cyanine 3 (CD8+) + cyanine 3.5 (Foxp3) + cyanine 5 (CD20+); (J) DAPI + cyanine 3 (CD8+) + cyanine 3.5 (Foxp3) + cyanine 5 (CD20+) + fluorescein (CD3+); and (L) DAPI + cyanine 3 (CD8+) + cyanine 3.5 (Foxp3) + cyanine 5 (CD20+) + fluorescein (CD3+) + cyanine 5.5 (CD4+).
Figure 3.4 An example TMA core comparison of chromogen and fluorophore stained sections (A) Single section stained for CD3 with green with a haematoxylin nuclear counterstain (light blue); (B) the adjacent multiplex sections for green stained showing CD3 positive cells (green) present along with CD8 positive cells stained red, FoxP3 positive cells stained with DAB (brown); (C) shows the single stain slide, in this case stained using CD3 with fluorescein (light blue) with a DAPI nuclear counterstain (dark blue); (D) shows the multiplex counterpart.

### 3.2.3 Validation of multiplex IHC with multi-spectral imaging

To validate the multiplex method the study design involved: (1) Measuring variation in TIL density measurements in sequentially stained sections. (2) Comparing the repeatability (intra-assay variation) and reproducibility (inter-assay variation) of TIL density measurements obtained in single versus multiplex stained replicates prepared on the same or different days (experimental design summarised in Figures 3.1 and 3.2). (3) Comparing TIL density measurements in single versus multiplex chromogen stained slides. (4) Comparing TIL measurements in single versus single and single versus multiplex fluorophore stained slides. (5) Comparing the chromogenic versus fluorescent staining by the Vectra system (Figure 3.5).
3.2.4 Statistical analysis

Spearman rank correlation (non-parametric) was used to study the concordance of positive cell counts in single compared to multiplex stained sections. Fisher’s r to z transformation was used to generate Z scores in order to compare the different correlation coefficients for each marker when needed. Bland-Altman plots were used for more detailed analysis of the levels of agreement and to calculate systematic differences between measurements (bias) obtained for single versus multiplex stained sections. Chi squared test was used to compare groups for statistical differences. Repeatability and reproducibility was assessed as coefficient of variation (CV) using the mean of three replicate values. All statistical analyses were carried out in GraphPad Prism 6.0 (La Jolla, CA, USA). The level of significance was set at P=0.05.
Figure 3.5 The slide arrangement used for studying intra- and inter-assay variation. Coefficient of variation (CV) for CD3 (A), CD8 (B), FoxP3 (C) and multiplex (D) chromogen stained slides. Three slides of same colour above (no fill – day 1, dotted fill – day 2, solid fill – day 3) were used on each day to study intra-assay CV on the respective days, and two serial slides (border above) were used to study inter-assay CV.
<table>
<thead>
<tr>
<th></th>
<th>Total number of cells per core</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TMA core (20 cores)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pair 1</td>
<td>Pair 2</td>
<td>Pair 3</td>
<td></td>
</tr>
<tr>
<td>DAPI</td>
<td>Haem</td>
<td>DAPI</td>
<td>Haem</td>
<td>DAPI</td>
</tr>
<tr>
<td>Haem</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.6 Experimental design for comparing the chromogen and fluorophore staining. Cell counts in each core for sequential DAPI and haematoxylin (haem) counterstained slides were evaluated.

### 3.3 Results

#### 3.3.0 Variation in sequentially stained sections

There was a highly significant correlation (P<0.0001) between the number of positive cells scored in different sections 3 µm, 6 µm and 9 µm apart for the three markers assessed using chromogen staining (Figure 3.7). The z transformation scores were not significantly different for the results obtained when sections 3 µm to 6 µm apart were compared for all markers. However, there was a significant difference between the results obtained for sections 3 µm versus 9 µm apart for FoxP3 (P=0.002) and CD8 (P=0.05) but not CD3 (P=0.39). Table 3.2 summarises the results from the Bland-Altman analyses of the level of agreement between scores (bias). The bias on the Bland-Altman plots increased with the distance between sections (Table 3.2). Comparison of the Bland-Altman scores for single versus single and single versus multiplex stained slides of equivalent distances showed no significant differences (P=0.64; Table 3.3). Subsequent experiments were limited to sections a maximum of 6 µm apart for chromogenic and 3 µm apart for fluorescent markers.
Each data point is the number of positive cells for a marker in a TMA core. The TMA comprised 20 cores from lymphoid tissues. The relationships are shown between the number of positive cell counts obtained for serial single sections stained for CD3 (A), CD8 (B) and FoxP3 (C). $R^2$ values for all plots were highly significant ($P<0.001$) showing a high degree of agreement between single section compared cell counts. Z-scores (generated from the $R^2$ values in order to compare the values for the three markers) were not statistically significant for CD3 and CD8 but were significant for FoxP3 sections 9 μm apart ($P=0.002$).
Table 3.2 Bland-Altman bias data for single marker stained section

<table>
<thead>
<tr>
<th>Marker</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD3</strong></td>
<td></td>
</tr>
<tr>
<td>Serial section</td>
<td>9.07</td>
</tr>
<tr>
<td>Two sections apart</td>
<td>14.66</td>
</tr>
<tr>
<td>Three sections apart</td>
<td>29.86</td>
</tr>
<tr>
<td><strong>CD8</strong></td>
<td></td>
</tr>
<tr>
<td>Serial section</td>
<td>5.82</td>
</tr>
<tr>
<td>Two sections apart</td>
<td>1.74</td>
</tr>
<tr>
<td>Three sections apart</td>
<td>47.21</td>
</tr>
<tr>
<td><strong>FoxP3</strong></td>
<td></td>
</tr>
<tr>
<td>Serial section</td>
<td>13.32</td>
</tr>
<tr>
<td>Two sections apart</td>
<td>21.20</td>
</tr>
<tr>
<td>Three sections apart</td>
<td>40.77</td>
</tr>
</tbody>
</table>

Table 3.3 Bland-Altman bias data for single versus multiplex stained section

<table>
<thead>
<tr>
<th>Marker</th>
<th>Single comparison (%)</th>
<th>Multiplex comparison (%)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>9.07</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>5.82</td>
<td>4.08</td>
<td>0.64</td>
</tr>
<tr>
<td>FoxP3</td>
<td>21.20</td>
<td>26.4</td>
<td></td>
</tr>
</tbody>
</table>

* Chi-square test

### 3.3.1 Repeatability/reproducibility of the multiplex method

Intra- and inter-assay variability was assessed as shown in Figure 3.4 and the results are summarised in Table 3.4. The similar CVs show that the multiplex staining was as repeatable and reproducible as the single marker staining method for both chromogens and fluorophores.
Table 3.4 Intra- and inter-assay variability in TIL counts for single and multiplex chromogenic and fluorescent stained sections

<table>
<thead>
<tr>
<th>Chromogenic staining</th>
<th>CD3+</th>
<th>CD8+</th>
<th>FoxP3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>S</td>
<td>M</td>
<td>P *</td>
</tr>
<tr>
<td>Intra-</td>
<td>20.8 ±6.1%</td>
<td>23.0 ±3.5%</td>
<td></td>
</tr>
<tr>
<td>Inter-</td>
<td>24.7 ±11.7%</td>
<td>25.6 ±11.1%</td>
<td>0.97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluorescent staining</th>
<th>CD3+</th>
<th>CD8+</th>
<th>CD4+</th>
<th>FoxP3+</th>
<th>CD20+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>S</td>
<td>M</td>
<td>S</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>Intra-</td>
<td>8.4 ±2.3%</td>
<td>10.4 ±3.2%</td>
<td>7.8 ±2.2%</td>
<td>9.5 ±2.9%</td>
<td>20 ±10%</td>
</tr>
</tbody>
</table>

S- singleplex immunohistochemistry, M – multiplex immunohistochemistry, ± - standard error of the mean (SEM)
Intra-assay variation – the coefficient of variation from three repeated stains on the same day.
Inter-assay variation – the coefficient of variation from two repeated stains on different days.
* Chi-square test
3.3.2 Comparison of single versus multiplex chromogen stained slides

Examples of staining are given in Figure 3.3. Comparisons for the three TIL markers between the single versus multiplex (triplex) and single versus single stained slides showed a consistently highly significant Spearman correlation (P≤0.0001 for all). The Spearman correlation r values for single versus single and single versus multiplex sections were ≥0.93 for CD3+, CD8+ and FoxP3+ (Figure 3.7, and Table 3.5).

3.3.3 Comparison of single and multiplex fluorophore stained slides

Figure 3.3 shows examples of fluorophore staining. Similar to the chromogen validation single versus single and single versus multiplex (quinplex) comparisons showed consistently highly significant correlations (P<0.0001). The Spearman correlation r values for single versus single and single versus multiplex comparisons for CD8 (0.97, 0.94), CD3 (0.92, 0.95), CD20 (0.88, 0.85), FoxP3 (0.75, 0.74) and CD4 (0.59, 0.58) are shown in Figure 3.9 and Table 3.6.
Figure 3.8 Comparisons of TIL counts in single versus multiplex chromogen stained slides. The scatter plots compare the absolute number of CD3 (A), CD8 (B) and FoxP3 (C) positive cells in 82 cores from 40 patients. CD3 and CD8 were sequential section comparisons while FoxP3 was separated by 6 μm from the comparator. Each point represents a single core for which two total positive cell counts are calculated across two sequential sections. Strong Spearman correlations (P<0.0001) are seen throughout.
Table 3.5 Spearman correlation values for the chromogenic triplex validation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marker</th>
<th>Single vs. single</th>
<th>Single vs. multiplex</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>Vector Red</td>
<td>0.96</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>Vina Green</td>
<td>0.98</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>FoxP3+</td>
<td>DAB</td>
<td>0.95</td>
<td>0.94</td>
<td>0.97</td>
</tr>
</tbody>
</table>

*Chi-square test

Table 3.6 Spearman correlation plots for the fluorescent quinplex validation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marker</th>
<th>Single vs. single</th>
<th>Single vs. multiplex</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>Fluorescein</td>
<td>0.92</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>Cyanine 3</td>
<td>0.97</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>CD4+</td>
<td>Cyanine 5.5</td>
<td>0.59</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>FoxP3+</td>
<td>Cyanine 3.5</td>
<td>0.75</td>
<td>0.74</td>
<td>0.99</td>
</tr>
<tr>
<td>CD20+</td>
<td>Cyanine 5</td>
<td>0.88</td>
<td>0.85</td>
<td></td>
</tr>
</tbody>
</table>

*Chi-square test
Figure 3.9 Comparison of TIL counts for single versus multiplex fluorophore stained slides. The scatter plots compare the absolute number of CD3 (A), CD4 (B), CD20 (C), FoxP3 (D) and CD8 (E) positive cells in 40 cores from different cancers. Sequential sections were 3 μm apart. Each point represents a single core for which two total positive cell counts were calculated across the two sequential sections. Highly significant Spearman correlations (P<0.0001) are seen throughout.
3.3.4 Chromogenic versus fluorescent staining

Paired sequential TMA sections counter stained with either DAPI or haematoxylin were used to evaluate cell counts by the Vectra system (Figure 3.6). Three sections for each marker were used. The Spearman correlation for the paired DAPI versus haematoxylin TIL count comparisons was $r = 0.88$ ($P<0.0001$; Figure 3.10). Mean, and median CVs between the three DAPI and haematoxylin stained sections were 4.43%, 4.08% and for the three haematoxylin sections 8.20%, 5.44% respectively. The intra-assay CVs (n=3) for the chromogenic and fluorescent staining were 23%, 15.7% and 20.8% for CD3+, CD8+ and FoxP3+ cells. For multiplex fluorescent staining intra-assay CVs were 10.4%, 9.5%, 22%, 12% and 15% for CD3+, CD8+, CD4+, FoxP3+ and CD20+ cells respectively (Table 3.4).

![Total cell counts](image)

Figure 3.10 Correlation of absolute TIL cell counts in TMA cores. Sections counterstained with DAPI or haematoxylin correlated strongly ($r = 0.88$, $P<0.0001$)
3.4 Discussion

There is increasing evidence for the immunogenicity of tumours, and that assessment of an “immune score” may have prognostic significance (Galon et al. 2012). Moreover, recent clinical data with immune checkpoint inhibitors suggest that patient selection based on the expression of PD-L1 in the tumour microenvironment may be required to guide treatment (Zitvogel and Kroemer 2012). Whilst the development of multiplex IHC permits analysis of antigen co-localisation in tissues for clinical application, this approach needs to be comparable to current single stain IHC methods. This study compared single antigen and multiplex quantification of TILs in TMA cores by IHC. Automated image analysis was used to ensure unbiased and reproducible scoring, and also allowed for assessment of multiple chromogens/fluorophores.

This is the first study to compare, quantitatively, serial single IHC stained sections using automated image analysis. Comparing single stain IHC across varying depths (3 μm, 6 μm and 9 μm) showed a consistently highly significant correlation for all markers under consideration. However, the observed bias increased as the distance increased. This reflects a change in tumour architecture and its effect on lymphocyte quantification. It is now well recognised that heterogeneity exists within tumours (Burrell et al. 2013). The data from this analysis provided acceptable parameters for comparing sections in this and in future studies. Pertinently, it demonstrates a change in TIL count with each subsequent section does not support the current use of different single stained slides to evaluate different antigens in the same patient. Interestingly, the data showed similar cell count variations in both single and multiplex chromogen and fluorophore stained slides. Also, the reproducibility of the multiplex method was shown by comparing the CVs for repeated single and multiplex staining. This study showed no difference in the CVs for repeat single versus multiplex stained sections. This is particularly important as it suggests that for a multi-marker study, a multiplex method will be less variable than repeated single marker staining.

A comparsion of the total TIL counts for single versus multiplex stained slides yielded high correlation coefficients for all the markers, and for both chromogens and fluorophores. Another group compared the Z-score (standard scores), which compares the standard deviation to the population mean, between multiplex stained
slides and reported an $R^2$ of 0.80 (Huang et al. 2013). This method depends on the data being normally distributed, but does not compare two observations directly. The significant correlation coefficients observed despite our approach of cell counting, rather than comparing qualitatively expression profiles/intensity of the stained of the cells, further supports the validity of the multiplex method.

In this study antibody shielding was controlled for by using optimised antibody concentrations. The issues related to the chromogens are more difficult to control for as there are few available standardised chromogens available commercially. For chromogenic multiplex IHC, others reported a role for DAB in preventing cross reactions with subsequent immunoreagents in multiplex IHC (Sternberger and Joseph 1979). Furthermore, this protective function may also serve to obscure closely related antigens (Valnes and Brandtzaeg 1984). Comparing the performance of chromogenic single and multiplex staining demonstrated a strong relationship between the absolute cell counts across both methods. However, the CV between sequentially stained slides for both methods showed a lower mean CV for fluorescent staining. In addition the intra-assay CV in the reproducibility experiment was less than for the multiplex method for both chromogens and flourophores. The above observations demonstrate the superiority of the fluorescent method. This may be due to less antigen shielding with the Tyramide-signal amplification fluorophore given the narrow spectral signatures rendering this approach optimal for the measurement of multiple antigens.

In the present study the enumeration of cells was the primary focus. However, the use of multiplex IHC for H-scoring (McCarty et al. 1985) to evaluate the intensity at the same time as the density of expression of multiple markers in the same section is also feasible. Furthermore, the introduction of multiplex IHC in routine practice will improve efficiency, as it will reduce the turnaround time and provide more information. There is variation between IHC staining of specimens which might limit the approach of using a single specimen for generating spectral libraries. This is particularly important in fluorescent staining, which is known to be limited by tissue auto-fluorescence. In addition to proper IHC methodology involving endogenous enzyme and protein blockade, at present it is recommended that the spectral libraries generated from matched tissue and fluorescent based tyramide detection systems are used. This study demonstrates that up to four chromogenic (including haematoxylin)
and six fluorescent markers (including DAPI) are achievable and comparable to single stain IHC.

In summary, quantitative multi-marker enumeration of TILs is comparable to single stain IHC and reproducible. As multiplex staining provides additional information on the co-localisation of TILs and maximises the use of valuable and limited clinical samples, it should be used in preference to single marker staining for \textit{in situ} enumeration of TILs by immunophenotyping. It is recommended (based on current available platforms) that a chromogen marker is optimum for up to four marker detection, but for greater than four markers the fluorescent staining method should be used for multiplex IHC.
4.0 Differences in immune cell density and localisation in HPV positive and negative OPSCC.

4.1 Introduction

HPV positive and negative OPSCC have different molecular and clinical features (Fakhry et al. 2008, Cancer Genome Atlas 2015) with HPV positive disease having a clearly established better prognosis irrespective of treatment type. This difference is likely to involve multiple factors including differential radio/chemosensitivity and genetic heterogeneity (Ang et al. 2010, Kimple et al. 2013). HPV positive OPSCC are found more frequently at the tonsil and tongue base sub-sites in the oropharynx but it is unclear what factors underlie this relationship. It is possible that activation of local oropharyngeal immunity plays a role in limiting the spread of the disease and/or enhancing response to therapy.

A predominant infiltration of CD3 T lymphocytes is associated with a favourable prognosis in several cancer types (Gooden et al. 2011). However the type and functional status of the immune cells (e.g. CD8+ cytotoxic effector versus tumour promoting CD4+FoxP3+ T regulatory [Treg] cells) and/or the microenvironment localisation of different tumour infiltrating lymphocytes (TILs) can determine the balance between control or promotion of cancer (Fridman et al. 2012). Recent OPSCC TIL studies have produced inconsistent findings. Studies of 46 (Wansom et al, 2012) and 83 (Nasman et al, 2012) patients, reported T cell infiltration was associated with a good prognosis and the degree of T cell infiltration did not differ by HPV status. By contrast, other studies reported that higher CD8+ but not CD4+ T cell infiltration was associated with a good prognosis and the degree of infiltration was positively related to HPV status (Nordfors et al. 2013, Ward et al. 2013). Ward et al, 2013 investigated 270 OPSCC and found significant differences in T cell infiltration between HPV positive and negative tumours with higher levels associated with a favourable outcome in HPV positive patients.

OPSCC have both tumour and stromal elements which may interact to influence the biology of the cancer. Activation of the tumour stroma may drive tumour progression metabolically and/or influence inflammatory responses by modulating the balance of negative and positive immune controlling processes (Meseure et al. 2014). Interestingly, activation of the OPSCC stroma determined by smooth muscle actin (SMA) expression was associated with a poor prognosis although the
relationship with HPV status was not determined (Marsh et al. 2011). The stromal extracellular matrix can influence anti-tumour immunity by controlling the positioning and migration of T cells as seen in human lung tumours (Salmon and Donnadieu 2012). A recent study of OPSCC reported that high CD8+ T cell infiltration in the stroma was associated with a good prognosis although, surprisingly, HPV status did not predict a better clinical outcome in this group of patients (Balermpas et al. 2013).

There is currently interest in de-escalating the treatment of good prognosis OPSCC. Stratification based on HPV status alone may be too simplistic and underpins the need to identify additional biomarkers of outcome. Measurement of TILs is considered a promising avenue of research but requires increased understanding of the subtleties of tumour immunologic response (Jones 2014). Therefore, the aim of the study reported here was evaluate the differences in TIL density and localisation in HPV positive and negative OPSCC and observe for the relationship between TIL density and survival. The aim was addressed by investigating OPSCC from patients who were diagnosed between 2002 and 2011 and received radiotherapy. Tumour HPV status was determined using three methods and multiplex immunohistochemistry (IHC) was used to detect and enumerate different T cell populations in tumour and stromal regions.

4.2 Materials and methods

4.2.1 Patients:

A retrospective audit using a radiotherapy database at The Christie NHS Foundation Trust Hospital identified patients with a confirmed histological diagnosis of OPSCC. Patients were treated between January 2002 and December 2011 with radiotherapy as one or the only therapy modality. Patients treated with a palliative intent were excluded. Patient clinico-pathologic and outcome data were collected from the case notes and The Christie Head and neck assessment forms. The study obtained ethics approval from National Health Service (NHS) Health Research Authority (HRA) National Research Ethics Service (NRES) committee in the North West (reference number 03/TG/076). Individual patient consent was not required. Pre-treatment formalin-fixed paraffin-embedded (FFPE) blocks prepared at biopsy were requested.
4.2.1 HPV detection

As described in Section 2.2.2 patients with concordant HPV positivity or negativity were included in the study. A consort diagram for patient selection is shown in Figure 4.1.

![Consort diagram of patient selection for the study](image)

4.2.2 Multiplex TIL immunohistochemistry

FFPE sections 4 μm in thickness were deparaffinised in xylene and rehydrated through graded concentrations of alcohol. Following epitope retrieval (HIER) in a pressure cooker, slides were placed in a Biogenex i6000 autostainer and endogenous peroxidases blocked using 3% peroxide solution (ACROS Organics, Geel, Belgium).
The first multiplex staining step was DAB visualisation (brown) of FoxP3+ antigen. Protein block was achieved using a 10% casein solution (Vector laboratories, Burlingame, CA, USA) prior to staining with the primary antibody (FoxP3, clone 236A/E7 mouse monoclonal antibody [mAb; Abcam, Cambridge, UK], 1:40). Secondary antimouse EnVision® HRP detection system (Dako, Cambridge, UK) was subsequently used for DAB visualisation. In between stains, slides were transferred to high pH antigen retrieval solution and microwaved at 98°C for 10 min. For the second and third stains, following peroxidase and casein blocking, CD3 (mouse mAb, clone F7.2.38 [Dako], 1: 60) and CD4 (mouse mAb, clone 4B12 [Dako], 1:50) were visualised with the HRP chromogens ImmPACTTM VIP SK-4605 (purple) and Vector SG (blue-grey). The fourth step was Vector® Red SK-5100 visualisation (red) of CD8 antigen. Following blocking with normal horse serum solution, CD8 antibody (mouse mAb clone C8/144B [Dako], 1:60) was applied and secondary anti-mouse Ig alkaline phosphatase [ImmPRESSTM (Vector Laboratories, Inc. Burlingame, CA, USA) was subsequently used to visualise Vector red. Mouse IgG1 (Dako) was used as a negative control. Finally, following multiplex IHC, sections were counterstained with haematoxylin, dehydrated in graded concentrations of alcohol and cover-slipped in permanent, non-aqueous mountant.

4.2.3 Staining for smooth muscle actin (SMA)

Sections were cut and deparaffinised, and antigens retrieved as described above. This was followed by a peroxidase block and staining with primary antibody (mouse mAb anti-human SMA clone 1A4 [Dako], 1:50) or negative control reagent, visualisation reagent, and substrate-chromogen solution (DAB). Staining was scored using computer automated H-scores (see below) and by percentage positivity from 30 randomly selected stromal regions in the single slides. Mean scores for each tumour were calculated.

4.2.4 Multiplex IHC automated image analysis and scoring

For each slide, the Vectra automated multispectral imaging system (PerkinElmer, Massachusetts, USA) was used to perform both low (x4) and high (x20) power scans of 30 randomly selected tissue grids. Spectral libraries were generated from single stained slides using the Nuance FX multispectral imaging system software (PerkinElmer, Massachusetts, USA). Spectral libraries and the scanned multiplex
images were then loaded into inForm advanced image analysis software (PerkinElmer). The image analysis method illustrated in Figure 4.2 comprised: a) resolution of spectral properties of the multiplex stains; b) classification of regions as tumour, stroma and/or blank spaces; c) identification of different cell types and d) scoring based on the constituent spectral properties. The individual biopsy TIL density per region of interest (ROI; 3.5 x10^5 nm) was determined from 30 randomly selected ROIs of tumour or stromal areas for each section. The mean scores for the whole biopsy, tumour and stroma were generated. The median T cell density for each group was used to stratify patients into “high” or “low” TIL groups. Automated image analysis was used to quantify the percentage of stromal cells with positive SMA expression (percentage positivity). The staining intensity was classified as low, medium or high, and H-scores were generated by multiplying percentage positivity and staining intensity scores (Figure 4.3).

Figure 4.2 The method used for image analysis of multiplex biomarker stained sections. The steps involved: image acquisition and processing to obtain the actual multiplex stained image (A); composite imaging of stromal (green), tumour (red) and blank (blue) spaces (B); superimposing A and B (C); enumeration of cells using haematoxylin (D); classification of the quantified cells based on the spectral properties and multiplex staining for tumour infiltrating lymphocytes (E); and quantification of the different T cell populations in the different compartments as CD3+ (red), CD3+CD4+ (yellow), CD4+ (green) or negative for both markers (blue) (F).
Figure 4.3 Examples of different levels of SMA expression in tumour stroma. Examples show high (A), medium (B) and low (C) generalised expression. Also shown are examples of high (D), medium (E) and low (F) discrete patterns of expression. G is a pre-analysed image. H is the scored image from G with the different colours representing high (brown), medium (orange) and low (yellow) intensity staining patterns.

4.2.5 Data analysis

Image analysis data were exported to Microsoft Excel worksheets. Charts and data comparisons were performed using SPSS version 20.0 and GraphPad Prism. For the Box Plots shown, Shapiro-Wilk normality test was used to test for the distribution of the data. As the data were mostly not normally distributed, the Mann-Whitney test for comparing the data was used. Actuarial calculations of locoregional control (LRC) and overall survival (OS) were obtained using the Kaplan-Meier method. Univariate analysis was compared using the Log Rank (Mantel-Cox) method. Chi-
squared test was used to compare categorical data and the threshold for statistical significance was 0.05.

4.3 Results

4.3.1 Patient characteristics

FFPE tumour pre-treatment biopsies were available from 218 OPSCC patients. Of these, 139 biopsies showed congruency of HPV positive or negative phenotyping by all three detection methods and had sufficient material for further analysis. Concordance rates for HPV detection or non-detection by the three methods was 78%. Subset comparism to larger cohort showed no significant differences in all the variables. All patients received radiotherapy as part of their treatment, 53% of the patients received only radiotherapy, 20% and 18% of the patients had radiotherapy in combination with surgery or chemotherapy respectively, while 9% of the patients had surgery followed by chemoradiotherapy. Figure 4.1 is the consort diagram for the study. The 5 year LRC and OS rates for the cohort were 66% and 52% respectively. The univariate survival analysis of the patient cohort stratified by different clinico-pathological features is summarised in Table 3.1. Smoking, pre-treatment haemoglobin, tumour stage, differentiation and HPV status were associated with LRC and OS. Figure 4.4 also shows the better LRC (P=0.004) and OS (P=0.0003) of HPV positive compared to negative tumours.

![Figure 4.4 Kaplan-Meier plots of overall survival (A) and locoregional control (B) in 139 patients with oropharyngeal cancer stratified by HPV status.](image-url)
Table 4.1 Univariate analysis of clinicopathologic characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Locoregional control</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.60 (0.72–3.54)</td>
<td>0.24</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤58 (median)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>&gt;58</td>
<td>0.79 (0.40–1.53)</td>
<td>0.48</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>0.51 (0.26–0.90)</td>
<td>0.04</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>≤14</td>
<td>0.24 (0.69–0.86)</td>
<td>0.001</td>
</tr>
<tr>
<td>&gt;14</td>
<td>0.45 (0.19–1.06)</td>
<td>0.96 (0.49–1.88)</td>
</tr>
<tr>
<td>AJCC stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (1, 2, 3)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>High (4)</td>
<td>0.45 (0.23–0.87)</td>
<td>0.01</td>
</tr>
<tr>
<td>Tumour differentiation</td>
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<td></td>
</tr>
<tr>
<td>Well</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>0.26 (0.10–0.66)</td>
<td>0.002</td>
</tr>
<tr>
<td>Poor</td>
<td>0.24 (0.05–1.06)</td>
<td></td>
</tr>
<tr>
<td>HPV status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1</td>
<td>2.72 (1.41–5.23)</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HR=hazard ratio; CI=confidence intervals; Hb=haemoglobin; g/dL= grams per decilitre

4.3.2 Infiltration of T cells

The mean density of CD3+ T cells in the 139 biopsies (both tumour and stromal areas) was 5.2x10^3/ROI of which 60% (3.1x10^3) were CD4+ and 29% (1.5x10^3) CD8+; only 0.02% (0.06x10^3) of the CD4+ TILs were FoxP3+. HPV positive compared to negative OPSCC had significantly more CD3+ (P<0.0001), CD3+CD4+ (P<0.0001) and CD3+CD8+ (P<0.0001) but not CD4+FoxP3+ (P=0.1) TILs (Table 4.2, Figure 4.5). A similar pattern of significantly increased infiltration of CD3+, CD3+CD4+ and CD3+CD8+ but not CD4+FoxP3+ T cells was seen in both tumour and stromal regions of HPV positive compared to negative OPSCC (Table 4.2, Figure 4.5). There were 9.3 fold more CD3+CD8+ T cells in HPV positive compared to negative stromal regions of OPSCC (Table 4.2).
Table 4.2 The mean cell density of different T cell populations per region of interest

<table>
<thead>
<tr>
<th>Marker</th>
<th>All*</th>
<th>Tumour</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV positive</td>
<td>7.9 x 10^3</td>
<td>3.5 x 10^3</td>
<td>4.2 x 10^3</td>
</tr>
<tr>
<td>HPV negative</td>
<td>3.2 x 10^3</td>
<td>1.5 x 10^3</td>
<td>1.6 x 10^3</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1</td>
</tr>
<tr>
<td>CD3+CD8+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV positive</td>
<td>1.8 x 10^3</td>
<td>0.5 x 10^3</td>
<td>1.3 x 10^3</td>
</tr>
<tr>
<td>HPV negative</td>
<td>0.4 x 10^3</td>
<td>0.25 x 10^3</td>
<td>0.14 x 10^3</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD3+CD4+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV positive</td>
<td>5.1 x 10^3</td>
<td>2.3 x 10^3</td>
<td>2.4 x 10^3</td>
</tr>
<tr>
<td>HPV negative</td>
<td>1.6 x 10^3</td>
<td>0.8 x 10^3</td>
<td>0.9 x 10^3</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD4+FoxP3+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV positive</td>
<td>0.1 x 10^3</td>
<td>0.006 x 10^3</td>
<td>0.005 x 10^3</td>
</tr>
<tr>
<td>HPV negative</td>
<td>0.09 x 10^3</td>
<td>0.003 x 10^3</td>
<td>0.004 x 10^3</td>
</tr>
<tr>
<td>P</td>
<td>0.1</td>
<td>0.2</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Mean of 60 regions of interest each 3.5 x 10^5 nm (30 in stroma and 30 in tumour areas)

^74 HPV positive and 65 HPV negative oropharyngeal squamous cell carcinoma
Figure 4.5 Scatter plots showing the differential infiltration of the T cell subsets in HPV positive and negative oropharyngeal tumours (n=139). CD3+ (A), CD3+CD8+ (B), CD3+CD4+ (C) and CD4+FoxP3+ (D) densities were consistently significantly higher in HPV positive cancers.
4.3.3 TILs and clinical outcome

There was no difference in OS and LRC for the 139 patients when stratified by median CD3+ TIL density. Table 4.3 shows the distribution of patients according to high versus low overall CD3+ TIL density. Infiltration was significantly lower in higher American Joint Committee on Cancer (AJCC) prognostic staged tumours (P=0.02) and HPV negative tumours (P=0.02; Table 4.3). Sub-stratifying by HPV status showed that a high CD3+ T cell density was associated with significantly better OS in HPV positive (P=0.02) but not HPV negative (P=0.31) patients (Figure 4.6). There was no statistically significant difference in LRC for high versus low CD3+ T cell infiltration in either HPV positive or negative OPSCC. Further analysis of HPV positive OPSCC indicated that higher CD3+ T cell infiltration in the stroma (LRC P=0.03; OS P=0.01) but not the tumour (LRC P=0.75; OS P=0.15) regions. Interestingly, this improved OS (Figure 4.6) and LRC (Figure 4.7) was related to significant stromal, and not tumour, CD3+CD8+ but not CD3+CD4+ TILs in HPV positive OPSCC (Table 4.4).

Table 4.3 Clinico-demographic characteristics of patients in the high/low TIL groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TIL</th>
<th></th>
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<td></td>
<td>Low</td>
<td>High</td>
<td>P</td>
</tr>
<tr>
<td>HPV status</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
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<td>20</td>
<td>0.02</td>
</tr>
<tr>
<td>Positive</td>
<td>37</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
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<td>60</td>
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</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Sub-site</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tongue base</td>
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<td>44</td>
<td>0.85</td>
</tr>
<tr>
<td>Tonsil</td>
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<td>27</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤58</td>
<td>36</td>
<td>33</td>
<td>0.31</td>
</tr>
<tr>
<td>&gt;58</td>
<td>30</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
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</tr>
<tr>
<td>Never</td>
<td>0</td>
<td>2</td>
<td>0.16</td>
</tr>
<tr>
<td>Occasionally</td>
<td>38</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Heavy</td>
<td>19</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>12</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Ex</td>
<td>38</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>16</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>25</td>
<td>0.02</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>12</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>28</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>26</td>
<td>22</td>
<td>0.79</td>
</tr>
</tbody>
</table>
Figure 4.6 Kaplan-Meier plots of overall survival of 139 OPSCC patients stratified by T cell infiltration and tumour microenvironment compartment.
The panels show low versus high CD3+ T cell infiltration in all 139 (A), 74 HPV positive (B) and 65 HPV negative (C) tumours. Also, survival of HPV positive patients by CD3+ T cell infiltration in the tumour (D) and stromal (E) compartments respectively and survival of HPV positive patient in relationship to infiltration of CD3+CD8+ (F) and CD3+CD4+ T cells with (G) showing the significantly higher survival seen with higher CD3+CD8 T cell infiltration (P=0.05). H and I show HPV positive patient survival with infiltration of CD3+CD8+ T cells tumour and stromal compartments respectively. Stromal infiltration but not tumour infiltration was associated with significantly better survival (P=0.02).
Figure 4.7 Kaplan-Meier plots of loco-regional control for 139 OPSCC patients stratified by T cell infiltration and tumour micromenvironment compartment. The panels show low versus high CD3+ T cell infiltration in all 139 (A), 74 HPV positive (B) and 65 HPV negative (C) tumours. Also loco-regional control of HPV positive patients by CD3+ T cell infiltration in the tumour (D) and stromal (E) compartments, and of HPV positive patients in relationship to infiltration of CD3+CD8+ (F) and CD3+CD4+ T cells (G) respectively. HPV positive patient survival with infiltration of CD3+CD8+ T cells in tumour (H) and stromal (I) compartments respectively, stromal infiltration but not tumour infiltration was associated with significantly better loco-regional control (P=0.03).
<table>
<thead>
<tr>
<th>T cell infiltration</th>
<th>OS HR (95% CI)</th>
<th>P</th>
<th>LRC HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ Low</td>
<td>1.01 (0.44 – 2.29)</td>
<td>0.97</td>
<td>1.63 (0.70 – 3.80)</td>
<td>0.25</td>
</tr>
<tr>
<td>CD3+ High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV+ve Low</td>
<td>3.31 (1.20–9.12)</td>
<td>0.02</td>
<td>1.76 (0.87–8.23)</td>
<td>0.20</td>
</tr>
<tr>
<td>CD3+ HPV+ve High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV-ve Low</td>
<td>0.62 (0.62–1.38)</td>
<td>0.31</td>
<td>0.64 (0.19–2.10)</td>
<td>0.46</td>
</tr>
<tr>
<td>CD3+ HPV-ve High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV+ve (tumour)Low</td>
<td>2.06 (0.77–5.52)</td>
<td>0.15</td>
<td>1.08 (0.65–6.34)</td>
<td>0.75</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV+ve (stroma)Low</td>
<td>3.08 (1.22–7.80)</td>
<td>0.01</td>
<td>2.04 (1.16–9.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>CD3+ HPV+ve (stroma)High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV+ve Low</td>
<td>2.57 (1.05–6.26)</td>
<td>0.05</td>
<td>1.97 (0.97–5.7)</td>
<td>0.07</td>
</tr>
<tr>
<td>CD3+ HPV+ve High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV-ve Low</td>
<td>0.96 (0.56–4.3)</td>
<td>0.98</td>
<td>0.64 (0.1–5.3)</td>
<td>0.93</td>
</tr>
<tr>
<td>CD3+ HPV-ve High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV+ve (tumour)Low</td>
<td>0.17 (0.64–4.90)</td>
<td>0.26</td>
<td>1.60 (0.31–8.28)</td>
<td>0.57</td>
</tr>
<tr>
<td>CD3+ HPV+ve (tumour)High</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV+ve (stroma)Low</td>
<td>2.83 (1.23–7.16)</td>
<td>0.02</td>
<td>2.63 (1.30–8.30)</td>
<td>0.03</td>
</tr>
<tr>
<td>CD3+ HPV+ve (stroma)High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV+ve Low</td>
<td>2.09 (0.82–5.30)</td>
<td>0.11</td>
<td>1.56 (0.78–6.12)</td>
<td>0.26</td>
</tr>
<tr>
<td>CD3+ HPV+ve High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD3+ HPV-ve Low</td>
<td>0.76 (0.56–4.32)</td>
<td>0.45</td>
<td>0.65 (0.20–5.67)</td>
<td>0.96</td>
</tr>
<tr>
<td>CD3+ HPV-ve High</td>
<td>1</td>
<td></td>
<td>1</td>
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</tr>
<tr>
<td>CD4+ HPV+ve (tumour)Low</td>
<td>1</td>
<td>0.67</td>
<td>1</td>
<td>0.22</td>
</tr>
<tr>
<td>CD4+ HPV+ve (tumour)High</td>
<td>0.76 (0.23–5.34)</td>
<td>1</td>
<td>1.7 (0.72–4.25)</td>
<td></td>
</tr>
<tr>
<td>CD4+ HPV+ve (stroma)Low</td>
<td>0.69 (0.10–4.34)</td>
<td>0.56</td>
<td>0.98 (0.53–1.43)</td>
<td>0.45</td>
</tr>
<tr>
<td>CD4+ HPV+ve (stroma)High</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

### 4.3.4 Stromal activation

Stromal SMA expression (Figure 4.3) was significantly greater in HPV positive compared to negative tumours (Figure 4.8), but was not associated with clinical outcome (LRC P=0.34; OS P=0.79; Table 4.4). There was no relationship between the percentage positivity of SMA expression and stromal CD3+ TIL density in all
patients (Table 4.5). However a significant correlation was seen in HPV positive but not negative OPSCC stroma for CD3+ (P=0.01) and CD3+CD8+ (P=0.0005) T cell density (Table 4.6).

Figure 4.8 Box plots of H-scores (A) and percentage positivity (B) of SMA staining in the stroma of HPV positive and negative OPSCC showing significantly higher scores (P=0.03) and percentage positivity (P=0.04) in HPV positive tumours.

Table 4.5 Univariate analysis of SMA expression in OPSCC

<table>
<thead>
<tr>
<th>HPV status</th>
<th>SMA expression</th>
<th>Overall survival</th>
<th>P</th>
<th>Loco-regional control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>Low</td>
<td>2.63 (0.87 – 5.35)</td>
<td>1</td>
<td>1.34 (0.63–4.50)</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1</td>
<td>0.79</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV+</td>
<td>Low</td>
<td>0.63 (0.34 – 2.34)</td>
<td>1</td>
<td>0.78 (0.2–1.34)</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1</td>
<td>0.23</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV-</td>
<td>Low</td>
<td>0.87 (0.45 – 1.87)</td>
<td>1</td>
<td>0.45 (0.34–1.98)</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1</td>
<td>0.49</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.6 Spearman correlations for SMA expression versus TIL density

<table>
<thead>
<tr>
<th>TIL cell population</th>
<th>CD3+ Tcells (all)</th>
<th>CD3+ (HPV–ve)</th>
<th>CD3+ (HPV+ve)</th>
<th>CD3+CD8+ (HPV–ve)</th>
<th>CD3+CD8+ (HPV+ve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r value</td>
<td>-0.11</td>
<td>0.02</td>
<td>0.29</td>
<td>0.04</td>
<td>0.40</td>
</tr>
<tr>
<td>P value</td>
<td>0.20</td>
<td>0.86</td>
<td>0.01</td>
<td>0.78</td>
<td>0.0005</td>
</tr>
</tbody>
</table>
4.4 Discussion

The OPSCC cohort investigated here had similar OS and LRC rates and clinico-pathological prognostic factors as described previously by others (Gillison et al. 2008, Ang et al. 2010, Oguejiofor et al. 2013). To address potential inadequacies of assessing tumour HPV status in published studies, the three most common HPV detection methods (DNA PCR, DNA ISH, and p16 IHC) were used and only those patients with concordant results (78%) included for study. In the 139 patients with concordant results, 53% were classified as HPV positive, a value similar to that reported previously in OPSCC (Hama et al. 2014). It is pertinent to note that differing positivity rates have been reported globally with rates as low as 20% in the Netherlands to as high as 72% in parts of the USA (Rietbergen et al. 2013).

A strength of the study was the use of multiplex IHC and automated image analysis, which allowed for delineation of specific T cell populations and reproducible/representative quantitative scoring. This approach enabled not only the extraction of more information from a single slide, but also the observation of contextual relationships between detected antigens. The ability to localise the different immune cell phenotypes to different compartments thus adds a layer of complexity and novelty to the study of TILs in OPSCC. The study showed that patients with HPV positive OPSCC with high CD3+CD8+ T cell infiltration in stromal areas have the best clinical outcome, which highlights the importance of assessing the tumour microenvironment compartment in TIL studies.

Higher TILs were found in HPV positive compared to negative OPSCC. This finding agrees with several (Nasman et al. 2012, Nordfors et al. 2013, Ward et al. 2014) but not all (Wansom et al. 2012, Balermpas et al. 2014) studies. Significantly higher densities of CD3+, CD3+CD4+, CD3+CD8+ but not CD4+FoxP3+ T cells were detected in HPV positive compared to negative OPSCC as a whole in this study. A further key observation was that this significantly higher infiltration was seen in both tumour and stromal regions. It is apparent that evaluation of TIL density should be done in different tumour microenvironments (Fridman et al. 2012). Stromal cells have been reported to secrete cytokines which contribute to the recruitment of TILs to the tumour micro-environment (Gajewski et al. 2013). It is presumed that the immune cell infiltration into tumours occurs in response to tumour specific antigens and an attempt to control tumour growth and spread. However,
immune suppressive factors around the tumour periphery and/or expressed by the
tumours can also attract Treg cells able to inhibit otherwise potentially active
effector T cells. In this study, no difference was seen in the infiltration of Treg
(CD4+FoxP3) populations as stratified by HPV status although the numbers of these
cells detected were very small. Indeed, CD4+FoxP3+ T cells were often absent from
many of the scanned images (<4/per section) increasing uncertainly about the value
of quantitative comparisons. There is evidence by others that high levels of systemic
Treg cells are a positive prognostic marker in OPSCC (Khorramiirouz et al. 2014,
Lukesova et al. 2014). The approach used here of evaluating Treg cells in the local
tumour environment did not show any prognostic relationship. It is possible that
FoxP3+ may not mark all Treg cells and/or that other immune cells such as
macrophages or myeloid derived suppressor cells, that were not assessed, could act
to limit CD8+ T cell activity (Damuzzo et al. 2014, Caronni et al. 2015).

As expected and reported widely, HPV positive compared to negative OPSCC
patients had a significantly better OS (P=0.0003) and LRC (P=0.0037). In contrast
with other reports where T cell density correlated with survival in both HPV positive
and negative patients (Nasman et al. 2012, Wansom et al. 2012, Balermpas et al.
2014), no difference was found in outcomes when stratifying all patients by total
CD3+ T cell infiltration. The heterogeneity in the patients with oral cancers studied,
robustness of HPV detection and use of a variety of IHC methods may have
contributed to discordance between various published reports. However, T cell
infiltration was significantly associated with survival in HPV positive but not
negative OPSCC patients as reported in another study (Ward et al. 2013). In
addition, the observations suggest that the stromal infiltration of CD3+CD8+ T cells
is important in determining prognosis. This is consistent with a hypothesis that the
higher infiltration of CD8+ T cells in the stroma is a marker of an effective immune
response in HPV positive OPSCC contributing to improved outcome following
standard therapy. In cervical dysplasia, lesion regression has been associated with T
cell infiltration of the epithelium (Trimble et al 2010) but there is evidence that the
stroma is the first point of call for the effector immune cells (Kobayashi et al. 2004,
Trimble et al. 2010, Maldonado et al. 2014). Gene analysis of micro-dissected
specimens of pre and post vaccination dysplastic lesions showed an increase in genes
associated with effector immune cell phenotype, polarisation, function and activation in the stroma of post vaccination patients (Maldonado et al. 2014).

In addition to TILs, the activation status of the stroma was investigated using SMA expression by activated myofibroblasts. When present at sites of chronic inflammation myofibroblasts promote angiogenesis, extracellular matrix, growth factor and cytokine expression (Surowiak et al. 2007). SMA expression has been associated with a poor prognosis in several cancer types. In patients with SCC of the tongue, Kellermann et al observed higher SMA expression at the invasive margin significantly correlated with invasion into blood vessels, lymph node and neurons (Kellermann et al. 2007). Stromal features predicted disease mortality in oral cancer although the proportion of OPSCC and HPV positivity was not stated (Marsh et al. 2011). Our results showed a significantly higher expression of SMA in the stroma of HPV positive compared to negative OPSCC but there was no association with survival. The differing numbers, heterogeneity of pathology, therapy and follow up of patients with oral cancers studied, robustness of HPV detection and variations in IHC methodology may all have contributed to the discordance between the various published reports. It is pertinent to mention that the differences in treatment received by patients in the different groups might also account for some of the differences seen. Subset analysis of the effects in the different treatment groups are required in larger cohorts to test assess for the effects if present.

There are further levels that can influence immune control in cancer patients through co-stimulatory inhibitory pathways. Programmed cell death protein 1 (PD-1) is expressed on activated T-cells and its ligands (PD-L1 and PD-L2) on antigen presenting cells (APCs) and sometimes tumour cells. The infiltration of PD-1 expressing lymphocytes can be a marker of a favourable prognosis in HPV positive OPSCC (Badoual et al. 2013) but localisation of PD-L1 to the tumour stroma interface or other immune cell types (e.g. macrophages) might limit functional tumour infiltration (Lyford-Pike et al. 2013). Trials investigating the blockade of PD-1 receptors in a range of cancers (melanoma, colorectal cancer, non-small-cell lung cancer, renal cancer) have shown clinical activity (Gubin et al. 2014, Herbst et al. 2014, Powles et al. 2014, Tumeh et al. 2014). In this study the relationship between tumour microenvironment, expression of PD1, PDL1 and PDL2 by TILs, tumour cells and/or APC was not evaluated. It is hypothesised that the presence of
TIL in the tumour compartment maybe subject to the suppressive effects of the co-stimulatory factors. Future studies using a multiple marker approach could investigate this relationship.

In summary, there is a growing body of evidence for an underlying immune activity in OPSCC, which is more important for HPV positive compared to negative disease. The work reported here highlights the importance of evaluating its context-specific nature in relation to the immune cell localisation and functional orientation. As stated in the Introduction, the move towards patient stratification based on HPV status alone may be too simplistic with measurements of TILs considered a promising avenue for biomarker development (Jones et al, 2014). Our work supports this suggestion but, in addition, shows the need to assess microenvironment localisation.
5.0 The PD1-PD-L1 pathway and relationship to T cell density in oropharyngeal cancers

5.1 Introduction

The natural history of tumour development may be sculpted by the immune system with multiple mechanisms identified that facilitate immunological escape including down-regulation of HLA, altered composition of regulatory cells in the tumour microenvironment and up-regulation of suppressive immune checkpoint pathways (Dunn et al. 2002, Beatty and Gladney 2015). Recent clinical studies targeting co-inhibitory and co-stimulatory immune checkpoints such as cytotoxic T lymphocyte protein-4 (CTLA-4), programmed cell death 1 (PD-1) and its ligand (PD-L1) have demonstrated anti-tumour activity in several cancer types (Brahmer et al. 2010, Topalian et al. 2012, Wolchok et al. 2013, Powles et al. 2014).

Chronic activation of T-cells leads to an exhausted phenotype which is typically identified in tumour-infiltrating T cells. (Sakuishi et al. 2010) T-cell expression of PD-1 is indicative of this phenotype and signalling through its ligand, PD-L1, can attenuate signalling through the T-cell receptor (TCR) and lead to anergy/apoptosis and contribute to immune escape. The expression of PD-L1 by tumour infiltrating immune cells (including B and T lymphocytes, dendritic cells, and macrophages), vasculature, endothelium, malignant cells and in the associated stroma can provide for significant immune regulation (Dong et al. 1999, Freeman et al. 2000, Latchman et al. 2001, Tseng et al. 2001)(Riley and June 2005)(Dolan and Gupta 2014).

Expression of PD-L1 is inducible in responses to changes in inflammatory mediators in the tumour microenvironment such as interferon (IFN) γ and tumour necrosis factor (TNF)α making PD-L1 a dynamic and potentially problematic biomarker. It is perhaps unsurprising that there is heterogeneity in responsiveness to PD-1/PD-L1 monoclonal antibody (mAb) therapy in relation to expression of PD-L1 in the tumour microenvironment. (Chen and Han 2015) Indeed, up-regulation of PD-L1 during treatment may be indicative of therapeutic response to checkpoint inhibitor treatment (Tumeh et al. 2014).

In oropharyngeal squamous cell carcinoma (OPSCC), better clinical outcomes are reported in HPV+ compared to negative patients irrespective of treatment and this is
linked to differences in immune infiltrating T cells. (Oguejiofor et al. 2015) Persistent infection is the key risk factor in the natural history of HPV associated cancers (Castellsagué 2008) and in the oropharynx this may be facilitated by the presence of “immune privileged” sites provided by expression of PD-L1 in tonsil crypts (Lyford-Pike et al. 2013). However, higher expression of PD-L1 in HPV+ compared negative OPSCC has been reported but levels were not correlated to improved patient survival in either group. (Ukpo et al. 2013) Another study showed that improved survival can also be associated with increased expression of PD-1+ T cells in HPV positive OPSCC (Badoual et al. 2013).

The aim of this study was to investigate immune factors present in the HPV+ and negative OPSCC at the time of diagnosis and in particular assess the potential of PD-L1 expression as a biomarker for either prognosis or treatment selection. The approach used was multiplex immuno-fluorescent labelling to quantitate infiltration of tumours by CD8+ T-cells, their expression of PD-1, and the expression of PD-L1 on both tumours and CD68 cells (macrophages) correlated with, HPV status and outcome in a cohort of 125 OPSCC.

5.2 Materials and methods

5.2.1 Patient selection and HPV detection

Patient selection and HPV detection is described in Sections 4.2.0 and 3.2.1 respectively.

5.2.2 Multiplex TIL immunohistochemistry

FFPE sections 4 μm thick were stained using the ventana auto staining platform (Ventana Medical Systems, Tucson, Arizona, US). In this semi closed system (allowing for user interruption) was used for de-paraffinisation, epitope retrieval and endogenous peroxidases blockade and secondary antibody detection. A multiplex protocol involving an initial high pH (8.5) and subsequent low pH (6.0) in between fluorescent staining was used. The use of the tyramide-signal amplification (TSA) (Opal multiplex TSA system [PerkinElmer, Waltham, Massachusetts, US]) for fluorescent staining allowed for repeated staining steps. 100μl of antibody and opal
detection were applied by hand. The first antibody in the staining algorithm was rabbit monoclonal antibody (mAb) against PD-L1 (Cell Signaling, Danvers, Massachusetts, US, 1:200) detected using opal cyanine 5.5. An intervening antigen retrieval step followed each detection step. The second antibody was mouse mAb against CD8 antibody (clone C8/144B; Dako, Glostrup, Denmark, 1:60), which was detected using opal cyanine 3. The third and fourth were mouse mAbs against PD1 and CD68 (Abcam, Cambridge, UK; 1:50 and 1:200) detected by fluorescein and cyanine 3.5 respectively. The slides were then removed from the Ventana machine and submerged for 5 min, 3 times for a total of 15 min in EZ preparation in order to remove the oil film. They were then cover-slipped with Prolong aqueous mounting agent with DAPI (ThermoFisher Scientific, Waltham, Massachusetts, US) nuclear counterstain (Cell Signalling).

5.2.3 Multiplex IHC automated image analysis and scoring

Manual segmentation of tissue and stromal regions was done on selected tissue grids. The image analysis solution is described in Section 4.2.4. A PD-L1 scoring threshold of 5% of labeled cells in tumour specimens was used, which had been previously described as predicting patients who responded to anti-PD-L1 therapy (Herbst et al. 2014). Figure 5.1 shows examples of fluorophore multiplex staining.

Figure 5.1 Example of a section stained using multiplex fluorophore IHC. (A) actual image; (B) composite image (bright field); (C) composite image (fluorescent) with red (CD8+), green (CD68+), pink (PD-L1+) and magenta (PD1+) colours. De-convoluted of image (A) shows CD68+ (D), CD8+(E) PD-L1(F) and PD1(G) in single spectrum.
5.2.4 Data analysis

The data analysis method is as described in Section 4.2.5

5.3 Results

5.3.1 Patient characteristics

125 patients showed congruency of HPV positive or negative phenotype by three detection methods and had sufficient material for analysis. Samples from 14 patient had insufficient material for analysis. Of the remaining patients 76 (60%) were positive for HPV and 49 (40%) were HPV negative. Patient characteristics of this subgroup were not significantly different from the previous analyses as shown in Section 4.3.1.

5.3.2 CD8 T cell infiltration

Consistent with the previous study (Chapter 4), there was a higher CD8+ T cell density in HPV positive compared to negative OPSCC in both tumour (P=0.03) and stromal (P=0.02) areas. There were no significant differences in the densities of CD8+PD1+ T cells between HPV positive and negative OPSCC in either tumour or stromal sites, ). However, there were more CD8+PD-1+ T cells in HPV negative tumours in stroma and/ or tumour areas. This might be important as CD8+ T cell stromal densities link best to improved outcome. (Table 5.1). The higher proportion of CD8 T cells subject to inhibitory activity of PD-L1 in HPV negative patients might explain the relationship between CD8+ T cell densities in stromal regions to better outcome in the HPV positive patients.

5.3.3 PD-L1 expression in OPSCC

The mean (±SEM) OPSCC expression of PD-L1 was 4.22 ± 1.0 % The PD-L1 expression greater than 5% positivity across the tumour cohort irrespective of HPV status was 20%, with 26% and 16% positivity for HPV negative and positive tumours respectively. There was a higher mean expression in HPV negative (6.1± 2 %) compared to positive tumours (3.1± 1 %), which was significantly different by Mann-Whitney U test (P=0.01). Stratifying PD-L1 expression by site of expression, (stroma vs. tumour test) showed a higher PD-L1 expression in the tumour regions. However HPV negative tumours showed significantly higher PD-L1 expression at
stromal sites when compared with HPV positive tumours (P=0.01; Table 5.1). The data show that the higher PD-L1 expression in HPV negative tumours results from increased stromal expression of PD-L1. This pattern of expression would be consistent with it interfering with the function of CD8+PD1+ T cells in this location. A potential source of PD-L1 expression could be through infiltrating macrophages and this was analysed by analysing the CD68+ PDL1 expression in the OPSCC.

### 5.3.4 CD68 infiltration and PD-L1 expression

There was a higher density of CD68 cells in the HPV positive compared negative OPSCC in the tumour areas (P=0.01, Mann-Whitney U) and higher but not significant differences for the stromal regions (Table 5.1). Overall, 16% of the CD68 cells expressed PD-L1 in HPV negative compared with 7% in the HPV positive OPSCC. Interestingly, CD68+PD-L1+ stromal densities were significantly higher in HPV negative compared to HPV positive OPSCC (P=0.005, Mann-Whitney U). This is consistent with higher expression of PD-L1 observed in HPV negative compared with HPV positive OPSCC (Table 5.1) as due to PD-L1 expression on macrophages in the stroma. Figures 5.2 and 5.3 shows examples of staining of HPV positive and negative tumours.

![Staining examples](image)

**Figure 5.2 Example staining of HPV positive tumour.**

Actual images, segmented into (A) tumour (red) and (B) stroma (green) and (C) composite image. The composite image shows yellow (CD8+), pink (CD8+PD1+), red (CD68+), magenta (CD68+PD-L1+) and green (PD-L1+) cells.
Figure 5.3 Example staining of HPV negative tumour. Actual image, image segmented into (A) tumour (red) and (B) stroma (green) and (C) composite image. The composite image shows yellow (CD8+), pink (CD8+PD1+), red (CD68+), magenta (CD68+PD-L1+) and green (PD-L1+) cells.

Table 5.1 The mean cell density or expression of different T cell populations

<table>
<thead>
<tr>
<th>Immune cell type</th>
<th>HPV status</th>
<th>All</th>
<th>Tumour</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median cell density of T cells per ROI (3.5 x 10^5 mm^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>HPV positive</td>
<td>14.7</td>
<td>9</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>HPV negative</td>
<td>9.6</td>
<td>6.2</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>CD8+PD1+</td>
<td>HPV positive</td>
<td>2.8</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>HPV negative</td>
<td>2.2</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.1</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Mean percentage expressing of PD-L1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-L1+</td>
<td>HPV positive</td>
<td>3.1</td>
<td>2.3</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>HPV negative</td>
<td>6.1</td>
<td>3.2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.01</td>
<td>0.20</td>
<td>0.01</td>
</tr>
<tr>
<td>Median cell density of macrophages per ROI (3.5 x 10^5 mm^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td>HPV positive</td>
<td>46</td>
<td>34</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>HPV negative</td>
<td>35</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.08</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>CD68+PD-L1+</td>
<td>HPV positive</td>
<td>3.1</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>HPV negative</td>
<td>5.6</td>
<td>1.9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.004</td>
<td>0.4</td>
<td>0.005</td>
</tr>
</tbody>
</table>

In the previous chapter, a higher density of CD8+ T cells in the stroma was associated with better outcome (HPV positive). The level of PD1 activation and
potential interaction of these cells with the PD-L1 ligand expressed by either macrophages or tumour cells could limit this influence. By contrast, HPV negative OPSCCs had lower CD8+ infiltration than HPV positive tumours and higher proportions of CD8+ PD1+ T cells and CD68+ PD-L1 + macrophages in their stroma. These combinations would be expected to be associated with a poor prognosis.

5.3.4 Survival

Kaplan-Meier analysis of overall survival or local regional control stratified by levels above or below the median for CD8, CD8PD1, CD68, CD68PD-L1 or PD-L1 populations showed no significant associations (Table 5.2).

Table 5.2 Univariate analysis of immune cell markers in all patients

<table>
<thead>
<tr>
<th>Markers</th>
<th>LRC (HR, 95% CI)</th>
<th>P</th>
<th>OS (HR, 95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1</td>
<td>0.67, 0.34–3.5</td>
<td>0.78</td>
<td>1.21, 0.51–2.8</td>
<td>0.65</td>
</tr>
<tr>
<td>CD8+</td>
<td>0.45, 0.54–4.5</td>
<td>0.87</td>
<td>1.03, 0.49–2.13</td>
<td>0.93</td>
</tr>
<tr>
<td>CD68+</td>
<td>1.4, 0.24–2.3</td>
<td>0.65</td>
<td>1.24, 0.59–2.60</td>
<td>0.55</td>
</tr>
<tr>
<td>CD68+PD-L1+</td>
<td>0.56, 0.34–2.6</td>
<td>0.89</td>
<td>0.68, 0.32–1.41</td>
<td>0.30</td>
</tr>
<tr>
<td>CD8+PD1+</td>
<td>1.1, 0.45–3.4</td>
<td>0.57</td>
<td>0.72, 0.34–1.53</td>
<td>0.40</td>
</tr>
</tbody>
</table>

In HPV positive OPSCC patients only the CD8 density in the stroma correlated with improved survival. There was a no association for the CD68 and CD68PD-L1 levels of the stroma to associate with better outcome suggesting a macrophage contribution in tumour control (Table 5.3). The majority of PD-L1 expression was on tumour cells in the HPV positive OPSCC. Interestingly, higher expression of PD-L1 on tumour cells was not associated with a worse outcome (P=0.06). There was no correlation between PD-L1 tumour expression and CD8 densities in the stroma or tumour areas. For CD68 infiltration there was a positive correlation with CD8 levels in tumour (P=0.01) but not stroma.
### Table 5.3 Univariate analysis of immune cell markers in all areas of HPV positive and negative patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Markers</th>
<th>LRC (HR, 95% CI)</th>
<th>P</th>
<th>OS (HR, 95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV +ve</td>
<td>CD8+</td>
<td>0.34, 0.23 – 1.67</td>
<td>0.23</td>
<td>0.46, 0.18 – 1.15</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>CD68+</td>
<td>0.56, 0.45 – 2.3</td>
<td>0.56</td>
<td>0.64, 0.23 – 1.72</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>PD-L1</td>
<td>0.67, 0.18 – 3.12</td>
<td>0.67</td>
<td>0.78, 0.25 – 2.43</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>CD68+PD-L1+</td>
<td>0.34, 0.23 – 1.6</td>
<td>0.56</td>
<td>1.16, 0.43 – 3.11</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>CD8+PD-1+</td>
<td>0.67, 0.56 – 1.05</td>
<td>0.07</td>
<td>0.62, 0.22 – 1.69</td>
<td>0.31</td>
</tr>
<tr>
<td>HPV –ve</td>
<td>CD8+</td>
<td>0.45, 0.23 – 1.6</td>
<td>0.34</td>
<td>0.64, 0.22 – 1.85</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>CD68+</td>
<td>0.23, 0.12 – 1.8</td>
<td>0.46</td>
<td>0.63, 0.21 – 1.81</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>PD-L1</td>
<td>1.6, 1.2 – 5.7</td>
<td>0.03</td>
<td>2.76, 1.02 – 7.43</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>CD68+PD-L1+</td>
<td>2.5, 0.86 – 6.7</td>
<td>0.07</td>
<td>3.01, 0.96 – 9.39</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>CD8+PD-1+</td>
<td>0.56, 0.34 – 2.4</td>
<td>0.54</td>
<td>0.62, 0.22 – 1.69</td>
<td>0.35</td>
</tr>
</tbody>
</table>

### Table 5.4 Univariate analysis of immune cell markers in tumour and stroma regions of HPV positive and negative patients

<table>
<thead>
<tr>
<th>Markers</th>
<th>Patients</th>
<th>LRC (HR, 95% CI)</th>
<th>P</th>
<th>OS (HR, 95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV +ve</td>
<td>CD8+</td>
<td>1.56, 0.45 – 3.4</td>
<td>0.34</td>
<td>1.25, 0.60 – 2.61</td>
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<tr>
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<td>0.76</td>
<td>1.14, 0.37 – 3.44</td>
<td>0.81</td>
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<tr>
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<td>PD-L1</td>
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<td>0.08</td>
<td>0.34, 0.10 – 1.07</td>
<td>0.06</td>
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<td>0.05</td>
<td>0.37, 0.15 – 0.93</td>
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<td>0.23</td>
<td>2.86, 0.37 – 3.44</td>
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<td>PD-L1</td>
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<td>0.76</td>
<td>1.62, 0.4 – 6.0</td>
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<td>0.94, 0.23 – 3.75</td>
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<td>3.10, 0.82 – 5.7</td>
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<td>PD-L1+</td>
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<td>4.0, 1.0 – 7.7</td>
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<td>0.04</td>
<td>4.6, 1.23 – 7.0</td>
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-ve – negative, +ve – positive, LRC – loco-regional control, OS – overall survival, HR – hazard ratio
By contrast in the HPV negative OPSCC patients, infiltration of CD68+ (P=0.04) especially CD68+PD-L1+ (P=0.01) and increased expression of PD-L1 (P=0.03) in the stroma were all associated with significantly improved survival (Figure 5.4). This suggest the hypothesis that control in HPV negative tumours might be driven by macrophage activity. Most PD-L1 expression was associated with CD68+ cells as the levels were significantly correlated.

Figure 5.4 Kaplan-Meier plots of overall survival. (A) High vs low CD8 T cell infiltration in HPV positive, (B) high vs. low CD8+ T cells in tumour and stroma of HPV positive tumours, (C) high vs. low CD68+ cell infiltration in HPV positive tumours, (D) high vs. low CD68+ T cells in stroma of HPV negative patients, (E) high vs. low CD68+PDL1+ cells in stroma of HPV negative tumours and (F) high vs. low PDL1+ cell infiltration in HPV negative patients.
5.3.5 Immune cell characteristics associated with improved survival

For HPV positive patients sub-stratifying by CD8+ median density into high vs. low groups showed that there was no statistically significant difference between PD-L1 and CD68+ expression and infiltration respectively (Figure 5.5). For HPV negative tumours sub-stratifying by CD68+ median density into high vs. low groups showed that there were significantly higher CD8+ (P=0.007), CD68+PD-L1+ (P<0.0001) cell densities and increased PD-L1+ expression (P<0.0001) (Figure 5.5C-E).
Figure 5.5 Box Plots showing low vs. high CD8+ T cell densities. Stratified by (A) PD-L1 expression and B) CD68+ cell density. C, D and E show significant differences in high vs. low CD68+ cell stratified by PD-L1 expression (P<0.0001), CD8+ density (P=0.007), and CD68+PD-L1+ (P<0.0001) densities respectively.
5.4 Discussion

In this study immune cell (CD8+ and CD68+PD-L1+) densities differed between HPV positive and negative OPSCC and higher densities was associated with better outcomes in HPV positive and negative patients respectively. This may reflect a different natural history of oncogenesis with altered immune control mechanisms apparent in the cancers at time of diagnosis. Furthermore, these differences at pre-treatment might predict the prevailing mechanisms post treatment.

At present it is well established that a predominant infiltration by TIL is linked to favourable outcomes in several cancer types (Gooden et al. 2011). Higher densities of CD8+ T cells in tumour and stroma compartments of HPV positive OPSCC and the relationship to patient survival has been described in this cohort (Section 4) and replicated in this study. The potential mechanism involves an active immune response (evident by increased stromal infiltration of CD8+ cells) which can be recruited following therapy. This model has been observed in pre-post treatment biopsies in melanoma where a stepwise accumulation of CD8+ initially at the invasive margin and centre of the tumour was described in patients responding to immune modulating agents (Tumeh et al. 2014).

In contrast, there was no difference in CD8+PD1+ T cell infiltration between HPV positive and negative patients and no relationship with patient outcomes. Interestingly there was a higher percentage of CD8+ cells in the stroma expressing PD-1 in contrast to tumour site. Others have described similar observations of significant differences in CD8+ T cell density with difference in CD8+PD1+ T cell infiltration between HPV positive and negative OPSCC tumours (Badoual et al. 2013). Similarly, Lyford-Pike et al found difference in PD1 expression on TILs and on T cells around inflamed tonsils (Lyford-Pike et al. 2013). This suggests induction and activation of PD1 on CD8+ in response to inflammation. Although PD1 is expressed after ligation of the T cell receptor (Agata et al. 1996), there are no data to support the function of PD1 in the absence of signalling via its ligand (PD-L1) (Keir et al. 2008). Therefore the density of CD8+PD1 cells alone is insufficient to comment on functionality. In this study there was an almost equal density of CD8+PD1+ in HPV positive and negative tumours, but higher expression of PD-L1 in HPV negative cancers suggesting possible inhibition of CD8+ function to a
greater degree in the HPV negative OPSCC. This might represent a mechanism for the improved prognosis of HPV positive patients.

In this study, the median expression of PD-L1 in all patients was ~ 5% across all regions (tumour or stroma). In their study evaluating predictive correlates to anti-PD-L1 antibody, Herbst et al demonstrated that the 5% positivity threshold predicted patients responding to therapy (Herbst et al. 2014). Furthermore, we observed about 20% of patients had ≥ 5% expression of PD-L1 with significantly higher expression of PD-L1 seen in HPV negative patients. In addition, Herbst et al found 19% and 28% PD-L1 prevalence (5% cut-off) on tumour cells and immune cells respectively in HNSCC (Herbst et al. 2014), but there was no stratification by HPV status. Another study using TMA sections observed higher PD-L1 expression in HPV positive patients with overall PD-L1 expression of 46% (Ukpo et al. 2013). Methodological differences in the study (use of different antibody) and/or the fluctuations in PD-L1 expression in response to differing stimuli may account for the observed differences. However, this also demonstrates non-uniform expression of PD-L1 in HNSCC, which has been reported (Strome et al. 2003, Tsushima et al. 2006).

In addition, there was a no association between PD-L1 expression on tumour cells and patient survival seen in the HPV positive patients. This may suggest the inhibition of cytotoxic T cell function in these patients. This is supported by the reports of others showing a lower density of CD8+ TILs with higher PD-L1 expression, although this was not linked to patient survival or stratified by HPV status (Cho et al. 2011). HPV negative patients had a significantly positive relationship with PD-L1 expression and patient survival. This is contradictory to the observation in HPV positive patients and also the suggested role played by PD-L1 expression on cancers. This observation might be related to the increased expression of PD-L1 known to occur at sites of inflammation to provide peripheral tolerance and prevent auto-immunity (Keir et al. 2008). Others have not reported any relationship between PD-L1 and patient survival in HPV positive or negative patients (Ukpo et al. 2013). Experience for the use of PD-L1 as a prognostic marker in cancers has been mixed (Thompson et al. 2004, Ghebeh et al. 2007, Zitvogel and Kroemer 2012, Tang and Heng 2013, Ukpo et al. 2013, Velcheti et al. 2014). Contextualisation of tumour and/or immune cell expression of PD-L1 with immune
cell infiltrate provides more information of the immune micro-environment. In a clinical trial of an anti-PD-L1 agent (MPDL3280A), Herbst et al observed increased immune cell infiltrates and increased in PD-L1 expression in patients with regressing lesions following therapy (Herbst et al. 2014).

It is suggested that in cancer the TIL responses may be attenuated by the proximity to immune inhibitory factors. PD-L1 expression on tumour cells promote T cell apoptosis in in-vitro and in-vivo models (Dong et al. 2002, Hirano et al. 2005), and might promote tumour growth and possibly an adverse prognostic profile. However, intrinsic and extrinsic factors are linked to PD-L1 expression on tumour cells. Intrinsic cellular changes in response to oncogenesis shown to induce PD-L1 expression include: 1) increased proliferation potential (Ghebeh et al. 2007); 2) increased signalling via signal transducer and activator of transcription 3 (STAT3) (Akbay et al. 2013); 3) increased signalling via extracellular regulated kinases (ERK) (Yamamoto et al. 2009); 4) increased signalling via epidermal growth factor receptor (EGFR) (Azuma et al. 2014); 5) inactivation of phosphatase and tensin homolog (PTEN) (Parsa et al. 2007); possibly other yet to be identified factors. Similarly, extrinsic factors inducing expression of PD-L1 involve immune cell signalling via toll like receptor (TLR) and interferon gamma (IFN-γ) signalling pathways (Loke and Allison 2003, Liu et al. 2007, Qian et al. 2008). Increased PD-L1 expression in a background of increased immune cell density will represent patients with an anti PD1/PD-L1 targetable phenotype. Thus PD-L1 expression, type and density of immune cells must be co-evaluated.

Furthermore, macrophage infiltration had no association with patient survival in HPV positive patients. In HPV negative patient increased infiltration of macrophages into the stromal compartment was associated with improved patient survival, particularly macrophages expressing PD-L1. CD68 as a single marker is insufficient to identify the different macrophage subtypes known to infiltrate tumours. In addition to cell surface markers, cytokine profiles are also needed. This study did not distinguish between pro-inflammatory (M1) or suppressive (M2) macrophage phenotypes. Despite differing functions both macrophage types are recruited to sites of inflammation. The overall effect of macrophages depends on the balance between both types.
Co-localisation of PD-L1 on CD68+ cells was associated with a higher infiltration in the HPV negative patients particularly in the stroma. Differential expression patterns of CD68+PD-L1 cells have been described (membranous, peripheral or diffuse). It is suggested that macrophage expression of PD-L1 serves as a homeostatic mechanism which inadvertently provides an immune privileged milieu encouraging tumour growth. However, macrophages (CD68+) have inherent functions unrelated to their expression of PD-L1 but rather dependent on the macrophage type and contribution of other factors. In this study, the higher infiltration of macrophages expressing PD-L1 was associated with an improved prognosis in HPV negative patients. This may suggest predominant infiltration of M1 type macrophages known to exert an anti-tumour effect. It is hypothesised that increased infiltration of macrophage expressing PD-L1 reflect increased immune activity in these patients. This suggestion is further strengthened by: 1) the observation of significantly higher cytotoxic T cell densities and 2) increased expression of PD-L1 in patients with higher macrophage densities. Increased PD-L1 expression may be related to increased secretion of interferon gamma (IFN-γ) by cytotoxic T cells and M1 type macrophages in these patients, which are known to increase PD-L1 expression.

Pre-treatment immune phenotype as described above may predict responses following therapy. Radiotherapy is common to the patients included in this study. Radiotherapy and its abscopal effects are suggested to be central to immune activity post treatment (Reynders et al. 2015). This is dependent on immunogenic cell death (following radiotherapy) and subsequent recruitment/modification of host immune responses (Apetoh et al. 2007, Obeid et al. 2007, Lugade et al. 2008, Gameiro et al. 2014). The success of this model is dependent on a “critical threshold” of CD8+ T cells and/or absence of a highly suppressive immune micro-environment (Formenti and Demaria 2013). In this study, HPV positive patients with higher CD8+ T cells in pre-treatment biopsies might predict patients responding with a favourable immunogenic profile post radiotherapy. Similarly, in HPV negative patients with less immunogenic tumours, increased recruitment of macrophages expressing PD-L1 suggest a favourable immune milieu that can be stimulated following radiotherapy and/or augmented with immunotherapy.
Studies in large patient groups with pre- and post-treatment biopsies demonstrating changes in immune cell density/phenotype following radiotherapy are required. At present there are differing immune profiles in HPV positive and negative patients (Figure 5.6). This study found no evidence to evidence to suggest a prognostic role for PD1/PD-L1 alone in HPV positive or negative OPSCC. However, co-localisation of CD68+ cells, particularly in HPV negative patients was prognostic. The data from this study suggest studies characterising PD1/PD-L1 expression must be carried out in the context of immune cell localisation preferably using a multiplex approach as it is apparent that it is more complex than simple observations may suggest.

Figure 5.6 Schematic of prognostic immune factors in OPSCC
6.0 Relationships between tumour hypoxia, HPV status and patient survival in OPSCC

6.1 Introduction

Hypoxia has widespread effects on tumour biology that ultimately lead to the selection of a more aggressive and treatment resistant phenotype (Graeber et al. 1996, Semenza 2000, Pennacchietti et al. 2003, Erler et al. 2004, Hill et al. 2009, Rouschop et al. 2010, Wang and Ohh 2010, Cairns et al. 2011, Pettersen et al. 2015). Avascularisation and/or disordered vascularisation in tumours are implicated in the development and persistence of tumour hypoxia (Jain 2005). Tumours exploit homeostatic mechanisms in their adaptation to hypoxia. Hypoxia inducible factor (HIF)-1 is a heterodimer transcription factor composed of an α unit which dimerise with a constitutively expressed β subunit. Several stimuli have been linked to HIF-1α regulation including: lowered cellular oxygen concentration (Semenza 2003, Carroll and Ashcroft 2005), growth factors (Fukuda et al. 2002), and loss of tumour suppressor genes (Bardos and Ashcroft 2004). HIF-1α and HIF-1β belong to a family of basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) transcription factors. Other members include HIF-2α or HIF-1α-like-factor (HLF) and HIF-3α which are expressed in a cell type specific manner compared to the ubiquitously expressed HIF-1α (Li et al. 2006). Therefore HIF-1α expression is used most commonly as a marker of hypoxia in several tissue types. The stabilisation by dimerisation of HIF-1α by HIF-1β to form a transcription factor complex and subsequent migration to the nucleus is central to this pathway. The HIF complex up regulates numerous hypoxia responsive genes, which are linked to widespread functions (Brown and Wilson 2004).

In head and neck cancers, hypoxia is linked with a poor prognosis (Nordsmark et al. 2005, Peridis et al. 2011). Furthermore, evidence for the benefit of hypoxia modification as a treatment strategy is established (Overgaard 2011). Therefore identification of patients with hypoxic tumours is important for future patient prognostic and treatment stratification. In addition, the improved prognosis observed in HPV positive OPSCC led to trials of treatment de-escalation. However, no all patients within larger groupings may share similar prognostic features as shown above. These patients will be to amenable to alternative therapies. This changing epidemiology transition in OPSCC means it is important to know whether known prognostic are for all specific aetiological groups.
HPV and hypoxia status are proven prognostic factors in head and neck cancer, but the relationship between the two is not clear. It was initially suggested that hypoxia is not important in HPV positive head and neck cancer as observed in the DAHANCA 5 trial (Toustrup et al. 2012). A 15 gene hypoxia classifier failed to predict improved benefit from hypoxic modification in HPV positive patients as was observed in the HPV negative patients. However, another study by the same group using $^{18}$F-labelled fluoroazomycin arabinoside PET observed no difference in the hypoxic fractions in HPV positive and negative patients.

In cell lines Sørensen et al observed similar relative radio-resistance and sensitisation to hypoxia modification in both HPV positive and negative cells (Sorensen et al. 2013). Also, others have reported a differential response of cell lines to hypoxia dependent on the HPV status (Hanns et al. 2015). These conflicting observations underpin the need further studies evaluating the prognostic significance of hypoxia in OPSCC in relation to HPV status.

This study, evaluated the prognostic significance of hypoxia markers HIF-1$\alpha$ and CA-IX in HPV positive and negative OPSCC using univariate and multivariate analyses. The study used the checklist for reporting recommendations for tumour marker prognostic studies (REMARK) guidelines (McShane et al. 2005, McShane et al. 2006, McShane and Hayes 2012). The study involved an initial biomarker evaluation. As the results showed a relationship between the hypoxia markers and patient prognosis in patients with both HPV positive and negative tumours, a second validation cohort was obtained in an attempt to confirm and strengthen the finding. The cohorts were then combined in a final analysis.

6.2 Materials and methods

6.2.1 Patient selection

An audit of the radiotherapy database at The Christie NHS Foundation Trust Hospital identified patients with a confirmed histological diagnosis of OPSCC. Patients were treated between January 1999 to December 2004 (cohort A) and from January 2005 to December 2011 (cohort B) in the validation cohort. Tissue block retrieval, data collation and scoring for cohort A was done by Mr Navin Mani as part of his MD study. All patients in the study received radiotherapy as one or the only therapy modality. Patients treated with a palliative intent were excluded. Patient
clinico-pathologic and outcome data were collected from the case notes and The Christie head and neck assessment forms. The study was approved by the National Health Service (NHS) Health Research Authority (HRA) National Research Ethics Service (NRES) committee in the North West (reference number 03/TG/076). Individual patient consent was not required. Pre-treatment formalin-fixed paraffin-embedded (FFPE) blocks prepared at biopsy were requested.

6.2.2 HPV detection

HPV detection methods are described in Section 2.2.1. For cohort (A) HPV detection was by p16 and HPV DNA ISH, positivity or negativity was either double positive or negative for both tests respectively. For validation cohort HPV detection was by p16, HPV DNA ISH and HPV DNA PCR, positivity or negativity was either triple positive or negative by the test respectively.

6.2.3 HIF-1α staining

Immunohistochemical staining of HIF-1α was performed on 4 μm tumour sections using a BONDmax autostainer (Leica Biosystems, Milton Keynes, UK). HIF-1α mouse monoclonal (BD biosciences, Erembodegem, Belgium), was diluted 1:50 in Leica antibody diluent (Leica Biosystems). The negative control was IgG1 (Dako, Cambridge, UK) diluted 1:20 in antibody diluent. Tumour sections were placed in the BONDmax autostainer, dewaxed, rehydrated, and underwent heat induced epitope retrieval for 40 min at 100°C (pH 9). A 3% solution of hydrogen peroxide was then applied for 10 min before adding the primary antibody or negative control for 15 min, polymer reagent (Leica Biosystems) for 8 min, and DAB for 10 min, then sections were counterstained with haematoxylin.

6.2.4 CA-IX staining

Immunohistochemical detection of CA-IX was also on the BONDmax autostainer (Leica Biosystems). The primary antibody was a CA-IX mouse monoclonal (Leica Biosystems), diluted 1:50 in Leica antibody diluent (Leica Biosystems). The negative control was IgG1 (Dako) diluted 1:20 in antibody diluent. FFPE sections 4 μm in thickness were placed in the BONDmax autostainer, dewaxed, rehydrated, and subject to heat induced epitope retrieval for 40 min at 100°C (pH 6). Hydrogen peroxide solution (3%) was applied for 10 min before adding the primary antibody
or negative controls for 15 min. Polymer reagent (Leica Biosystems) for 8 min and DAB for 10 min were then applied in sequence followed by counterstaining with haematoxylin.

6.2.5 Scoring

Batch-to-batch variation was assessed by choosing two sections with high and low HIF-1α/CA-IX expression and running additional sections from these biopsies with each batch. The scoring system was as follows: 0= no staining; 1= ≤ 10% nuclear staining; 2= > 10% to 29% staining; 3= ≥30% staining. 0-1 and 2-3 were grouped as low and high expression respectively. Nuclear staining was scored for HIF-1α and membranous staining for CA-IX (Silva et al. 2008, Douglas et al. 2013). Sections were assessed by two scorers one of whom is a pathologist. Others have used a combination of percentage scoring and staining intensity (H-score) to report on tumour hypoxia. Our use of percentage positivity was to limit the possibility of improper assessment of staining intensity known to occur (Jensen 2013).

Figure 6.1 (A) positive staining for CA-IX; (B) negative antibody staining of same section showing same region in A; (C) positive staining for HIF-1α and (D) negative antibody staining of same section showing same region in C.
6.2.6 Statistical analysis

Comparisons between patient clinico-pathological features and hypoxia markers of HPV positive and HPV negative patients were undertaken using t tests for continuous variables and Chi squared tests for categorical variables. Actuarial calculations of loco-regional control (LRC) and cancer specific survival (CSS) were obtained using the Kaplan-Meier method. Univariate analysis was compared using the log rank (Mantel-Cox) method. The threshold for statistical significance was 0.05.

6.3 Results

6.3.1 Cohort A (discovery)

Table 6.1 summarises the characteristics for both cohorts. There were 90 patients in cohort A with a median age of 58.5 years. 81% of the patients were males with a positive smoking history. 63% of the patients had stage IV disease as classified by the AJCC prognostic groupings. 69% and 48% of the tumours had low HIF-1α and CA-IX expression respectively. Forty-one patients with HPV positive and 40 with HPV negative tumours had enough tissue for HIF-1α and CA-IX analysis.

Table 6.2 shows a comparison of clinic-pathologic data for patients stratified by HPV status. Patients with HPV positive tumours were younger (P=0.002), less likely smoke (P=0.0036) and had lower expression of HIF-1α (P=0.008).

6.3.2 Univariate analysis in the discovery cohort

Table 6.3 summarises the findings of the univariate analysis in cohort A. There was a significant relationship between HPV status and CSS (P=0.0001; Figure 6.1A) and LRC (P=0.016; Figure 6.1B). There was no relationship between gender and smoking status and any outcome measure in both HPV positive and negative patients. Prognostic grouping using the AJCC criteria showed no association for better LRC (P=0.08) and a significant association for CSS (P=0.04) in the HPV negative patients. There was no relationship between prognostic grouping and outcome in HPV positive patients. Smaller tumours (T stage) had significantly better LRC and CSS in both HPV positive (P=0.002, P=0.001) and negative (P=0.0002,
P=0.002) patients (Table 6.3). Furthermore, CA-IX and LRC of HIF-1α showed a relationship for improved survival in HPV positive and negative patients in cohort A. In addition, low H1F-1α in HPV positive patients and CA-IX in HPV negative patients was not associated with improved CSS. There was a significant association between lower CA-IX (P=0.04) expression and CSS in HPV positive patients only (Figure 6.2 A-D).

6.3.3 Cohort B (validation)

In view of the relationships identified a second cohort was studied. There were 98 patients in this group with a median age of 58.5 years. 80% of the patients were males 74% of the patients had a positive smoking history. 63% of the tumours were stage IV disease as classified by the AJCC prognostic groupings. 61% and 67% of the tumours were classified as high HIF-1α and CA-IX respectively. 52% of the patients had HPV positive tumours (Table 6.1).

Stratification of patients by HPV status (Table 6.2) showed no significant difference in the median age in both groups. In both groups there was no difference in the percentage distribution of gender and AJCC prognostic grouping. There were a significant association of higher T stage tumours in the HPV negative patients (P=0.02). There was significantly fewer non-smokers in the HPV positive patients (P=0.02). In addition, the percentage of hypoxic tumours by HIF-1α was significantly higher in the HPV negative patients (P=0.01) but there was no difference in percentage CA-IX positivity between HPV positive and negative tumours. As in cohort A, patients with HPV negative tumours were older, smokers with larger tumours and higher HIF-1α expression.

6.3.4 Univariate analysis in the validation cohort (B)

There was a significant relationship between HPV status, CSS (P= 0.0006) and LRC (P=0.004; Figure 6.1C and D). There was no relationship between gender, smoking status and prognostic grouping and LRC or CSS for both groups. There was no significant differences in LRC for patients with low HIF-1α positivity in HPV positive and negative patients in cohort B. In addition, CSS showed no significant relationship with improved survival with low H1F-1α and CA-IX in HPV positive patients only (Figure 6.2, E-H).
6.3.5 Comparison of both cohorts

In Table 6.1 Chi squared comparisons of the percentage distribution of clinical-pathological factors showed no difference in age, gender, smoking, T stage, AJCC stage and HPV status were compared. However, cohort B had significantly more hypoxic tumours as detected by HIF-1α (P<0.0001) and CA-IX (P=0.03).

In cohort A there was a significantly higher numbers of smokers in HPV negative patients (P=0.003). However, in cohort B there was a significantly higher number of non-smokers in the HPV positive patients (P=0.02). The differences in the smoking patterns seem to reflect a decreasing percentage of smokers in the later cohort. HIF-1α expression was lower in the HPV positive patients in cohort A (P=0.008) and significantly higher in HPV negative patients in cohort B (P=0.01). In general a similar pattern of percentage distribution of the patient characteristics was observed in HPV positive and negative patients in both cohorts.

Table 6.1 Patient characteristics for both cohorts

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<td>38 (39)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>High</td>
<td>25 (31)</td>
<td>60 (61)</td>
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<tr>
<td>CA-IX</td>
<td></td>
<td></td>
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<tr>
<td>Low</td>
<td>41 (48)</td>
<td>30 (31)</td>
<td>0.03</td>
</tr>
<tr>
<td>High</td>
<td>44 (52)</td>
<td>68 (69)</td>
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Table 6.2 Patient characteristics for both cohorts stratified by HPV status

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Discovery (cohort A)</th>
<th>Validation (cohort B)</th>
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<tbody>
<tr>
<td></td>
<td>HPV positive n=45 (%)</td>
<td>HPV negative n=45 (%)</td>
<td>P</td>
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</tr>
<tr>
<td>Age (median)</td>
<td>57</td>
<td>63</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36 (80)</td>
<td>37 (82)</td>
<td></td>
<td>42 (82)</td>
</tr>
<tr>
<td>Female</td>
<td>9 (20)</td>
<td>8 (18)</td>
<td>0.79</td>
<td>9 (18)</td>
</tr>
<tr>
<td>Smoking status</td>
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<td></td>
<td></td>
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<td>Ever-smoker</td>
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<td>3 (7)</td>
<td>0.0036</td>
<td>37 (73)</td>
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<tr>
<td></td>
<td>31 (69)</td>
<td>41 (93)</td>
<td></td>
<td>14 (27)</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>14 (25)</td>
<td>4 (9)</td>
<td>0.085</td>
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<td>T2</td>
<td>20 (37)</td>
<td>19 (42)</td>
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<td>20 (39)</td>
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<td>5 (10)</td>
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<td>AJCC stage</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (4)</td>
<td>2 (4)</td>
<td></td>
<td>2 (4)</td>
</tr>
<tr>
<td>II</td>
<td>5 (11)</td>
<td>8 (18)</td>
<td></td>
<td>6 (12)</td>
</tr>
<tr>
<td>III</td>
<td>13 (29)</td>
<td>4 (9)</td>
<td></td>
<td>8 (16)</td>
</tr>
<tr>
<td>IV</td>
<td>25 (56)</td>
<td>31 (69)</td>
<td>0.11</td>
<td>35 (68)</td>
</tr>
<tr>
<td>HIF-1α</td>
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<td></td>
</tr>
<tr>
<td>Low (0, 1)</td>
<td>34 (83)</td>
<td>22 (55)</td>
<td></td>
<td>33 (65)</td>
</tr>
<tr>
<td>High (2, 3)</td>
<td>7 (17)</td>
<td>18 (45)</td>
<td>0.008</td>
<td>18 (35)</td>
</tr>
<tr>
<td>CA-IX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0, 1)</td>
<td>17 (42)</td>
<td>24 (54)</td>
<td></td>
<td>15 (29)</td>
</tr>
<tr>
<td>High (2, 3)</td>
<td>24 (58)</td>
<td>20 (46)</td>
<td>0.28</td>
<td>36 (71)</td>
</tr>
</tbody>
</table>
6.3.6 Survival

Figure 6.2 Kaplan-Meier plots of cancer specific survival (CSS) and loco-regional (LRC). Graphs show data for cohort A (A,B) and B (C,D). There were significantly improved survival outcomes in the HPV positive group (A [P=0.0001], B [P=0.016], C [P=0.0006] and D [0.0004]).

A. Cohort A HPV and CSS
- HPV Pos: 45 patients
- HPV Neg: 45 patients
- P = 0.0001
- HR 3.68, 95% CI 1.8 - 7.20

B. Cohort A HPV and LRC
- HPV Pos: 45 patients
- HPV Neg: 45 patients
- P = 0.016
- HR 3.04, 95% CI 1.5 - 6.07

C. Cohort B HPV and CSS
- HPV Pos: 51 patients
- HPV Neg: 47 patients
- P = 0.0006
- HR 3.64, 95% CI 1.73 - 7.64

D. Cohort B HPV and LRC
- HPV Pos: 51 patients
- HPV Neg: 47 patients
- P = 0.0004
- HR 4.15, 95% CI 1.89 - 9.09
Table 6.3 Univariate analysis of clinico-pathological variables in HPV positive and negative patients in cohort A

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Loco-regional control</th>
<th>Cancer specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male vs female</td>
<td>0.93</td>
<td>0.30–2.82</td>
</tr>
<tr>
<td>HPV Pos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male vs female</td>
<td>0.71</td>
<td>0.12–4.04</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (1-2) vs high (3-4)</td>
<td>0.19</td>
<td>0.08–0.44</td>
</tr>
<tr>
<td>HPV Pos</td>
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<td></td>
</tr>
<tr>
<td>Low (1-2) vs high (3-4)</td>
<td>0.09</td>
<td>0.02–0.44</td>
</tr>
<tr>
<td>AJCC stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (I-II) vs high (III-IV)</td>
<td>0.45</td>
<td>0.18–1.12</td>
</tr>
<tr>
<td>HPV Pos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (I-II) vs high (III-IV)</td>
<td>0.32</td>
<td>0.02–3.64</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No/ex vs current</td>
<td>1.04</td>
<td>0.44–2.42</td>
</tr>
<tr>
<td>HPV Pos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No/ex vs current</td>
<td>0.80</td>
<td>0.23–2.82</td>
</tr>
</tbody>
</table>
Table 6.4 Univariate analysis of clinico-pathological variables in HPV positive and negative patients in cohort B

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cohort B</th>
<th>Loco-regional control</th>
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<th>Cancer specific survival</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR 95% CI P</td>
<td></td>
<td>HR 95% CI P</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male vs. female</td>
<td>1.93</td>
<td>0.56 – 5.82 0.78</td>
<td></td>
<td>1.83</td>
<td>0.67 – 5.0 0.23</td>
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<tr>
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<td>0.62 – 4.04 0.60</td>
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<td>0.94</td>
<td>0.11 – 8.07 0.95</td>
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<td>T stage</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
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<td>0.08 – 0.94 0.02</td>
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<td>0.50</td>
<td>0.17 – 1.40 0.18</td>
</tr>
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<td>HPV Pos</td>
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<td>0.2 – 1.6 0.2</td>
<td></td>
<td>0.35</td>
<td>0.08- 1.50 0.16</td>
</tr>
<tr>
<td>AJCC stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td>0.68</td>
<td>0.38 – 1.67 0.23</td>
<td></td>
<td>1.45</td>
<td>0.41 – 5.04 0.55</td>
</tr>
<tr>
<td>HPV Pos</td>
<td>0.56</td>
<td>0.2 – 5.64 0.70</td>
<td></td>
<td>1.20</td>
<td>0.12- 11.0 0.87</td>
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<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
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<td>0.49 – 4.42 0.56</td>
<td></td>
<td>0.59</td>
<td>0.25 – 1.41 0.24</td>
</tr>
<tr>
<td>HPV Pos</td>
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<td>0.56 – 4.82 0.68</td>
<td></td>
<td>0.33</td>
<td>0.06 – 1.54 0.15</td>
</tr>
</tbody>
</table>
Figure 6.3 Kaplan-Meier plots of LRC and CSS for low vs. high CA-IX and HIF-1α in both cohorts.

In cohort A there was no significant relationship with improved LRC for low CA-IX (A) and HIF-1α (B) for both positive and negative patients. CA-IX showed a significant association in HPV positive patients (P=0.04) but not in HPV negative patients (C). HIF-1α showed a no relationship with improved CSS in HPV positive patients (D). In cohort B there was no association with low vs. high CA-IX LRC in HPV positive or negative patients (E). There was no significant relationship with improved LRC with low HIF-1α in HPV positive patients (F). CSS showed no association with low vs. high CSS in both CA-IX and HIF-1α in both HPV positive and negative patients (G and H).
6.3.7 Combined analysis

6.3.7.1 Patient characteristics
The percentage distribution of patient characteristics distributed by high versus low CA-IX and HIF-1α is shown in Table 6.5. There was a significantly higher distribution of high HIF-1α expressing tumours in the HPV negative patients (P=0.002). There was no difference observed for other patient clinical characteristics (gender, AJCC stage, smoking status) and for CA-IX expression.

6.3.7.2 Univariate analysis
Log-rank analysis in HPV positive and negative patients showed significant differences in LRC and CSS for T stage and HPV status only (Table 6.6). There was no association seen for gender, AJCC stage and smoking status. Figure 6.3 shows no difference in LRC for low versus high CA-IX and HIF-1α for HPV positive and negative patients. CSS for CA-IX showed significantly improved survival in HPV positive (P=0.04) and negative (P=0.04) patients. HIF-1α showed no association with patient survival (Figure 6.3).

6.3.7.3 Multivariate analysis
Multivariate Cox-regression analysis of clinico-pathological features (gender, T stage, AJCC stage, smoking status, CA-IX and HIF-1α) in HPV positive and negative patients are shown in Table 6.7. In HPV positive patients T stage was the only significant prognostic factor for LRC (P=0.05) and CSS (P=0.037). In HPV negative patients, CA-IX and T stage were associated with LRC and CSS (rank 1 and rank 2 respectively).
Table 6.5 The percentage distribution of clinicopathological factors for the combined group

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HIF-1α (%)</th>
<th></th>
<th>CA-9 (%)</th>
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<td></td>
<td>Low</td>
<td>High</td>
<td>P value</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>85</td>
<td>79</td>
<td>0.26</td>
<td>83</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>21</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>26</td>
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<td>17</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>41</td>
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<tr>
<td>3</td>
<td>27</td>
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<td>4</td>
<td>21</td>
<td>13</td>
<td>0.13</td>
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<tr>
<td>AJCC stage</td>
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</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
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<td></td>
<td>14</td>
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<td>3</td>
<td>20</td>
<td>17</td>
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<td>17</td>
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<tr>
<td>4</td>
<td>64</td>
<td>62</td>
<td>0.76</td>
<td>67</td>
</tr>
<tr>
<td>Smoking</td>
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<tr>
<td>Never</td>
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<td>12</td>
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<td>18</td>
</tr>
<tr>
<td>Ex</td>
<td>47</td>
<td>46</td>
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</tr>
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<td>Positive</td>
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<td>37</td>
<td>58</td>
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<td>53</td>
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</table>
Table 6.6 Univariate analysis of loco-regional control and cancer specific survival for the combined group

<table>
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<tr>
<th>Characteristics</th>
<th>Loco-regional control</th>
<th>Cancer specific survival</th>
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</thead>
<tbody>
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<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male vs. female</td>
<td>1.16</td>
<td>0.53 – 2.52</td>
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<td>HPV Pos</td>
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</tr>
<tr>
<td>Male vs. female</td>
<td>1.09</td>
<td>0.26 – 4.65</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (1-2) vs. high (3-4)</td>
<td>0.28</td>
<td>0.15 – 0.54</td>
</tr>
<tr>
<td>HPV Pos</td>
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<td></td>
</tr>
<tr>
<td>Low (1-2) vs. high (3-4)</td>
<td>0.17</td>
<td>0.05 – 0.51</td>
</tr>
<tr>
<td>AJCC stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low vs. High</td>
<td>0.69</td>
<td>0.36 – 1.3</td>
</tr>
<tr>
<td>HPV Pos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low vs. High</td>
<td>0.58</td>
<td>0.12 – 2.77</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV Neg</td>
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<td>No/ex vs. current</td>
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<td>Neg vs. Pos</td>
<td>3.52</td>
<td>2.09 – 5.92</td>
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Figure 6.4 Kaplan-Meier plots for the combined cohort. (A) LRC for CA-IX; (B) LRC for HIF-1α (C) CSS for CA-IX; and (D) CSS for HIF-1α showing significant differences in CSS for CA-IX (P=0.04) and HIF-1α (P=0.04) expression in HPV positive and negative patients.

Table 6.7 Cox-regression multivariate analysis in HPV positive and negative patients

<table>
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<th>HPV positive</th>
<th>Characteristics</th>
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<th>CSS</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Exp (B)</td>
<td>P</td>
<td>Rank</td>
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<td>10.4</td>
<td>0.037</td>
<td>1</td>
</tr>
<tr>
<td>T stage</td>
<td>18.5</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>HPV negative</td>
<td>CA-IX</td>
<td>64</td>
<td>0.001</td>
</tr>
<tr>
<td>T stage</td>
<td>24</td>
<td>0.01</td>
<td>2</td>
</tr>
</tbody>
</table>
6.4 Discussion

It is recognised that aetiological factors now present two or possibly more biologically distinct groups in OPSCC (HPV positive and negative). Hypoxia markers have been shown to be linked to prognosis in OPSCC (Swartz et al. 2015). Prognostic factors need to be evaluated within these broad aetiological groupings. Identification of biomarkers which stratify patients by prognosis within HPV positive and negative OPSCC groupings are needed. In this study there was an almost even split of patients by HPV status consistent with positivity rates between 44% to 57% depending on the HPV detection method in the United Kingdom (Wells et al. 2015). Others have described year on year increases in the incidence rates of 2.65% (Hammarstedt et al. 2007) mostly in younger patients (Shiboski et al. 2005), and higher prevalence rates for HPV positive OPSCC. This study did not see a rise in HPV positive OPSCC between both cohorts. These differences in observation may be linked to geography and possibly HPV detection methods.

As expected there was a relationship between HPV status and younger patient age in both cohorts, an observation that is well reported (Gillison et al. 2000, Shiboski et al. 2005). The later cohort had fewer smokers in the HPV positive group and an almost equal percentage of smokers in both the HPV negative group. In the earlier cohort there were more smokers in both HPV positive and negative patients. This finding reflects a transition with fewer smokers in the later years. The decreasing prevalence of smokers with an almost stable incidence of HPV negative patients may suggest the influence of other aetiological factor, possibly alcohol and/or genetic factors. There was a relationship between HPV status and HIF-1α expression with significantly lower HIF-1α expression in HPV positive tumours in both cohorts. The association was maintained in the pooled analysis despite differences in HIF-1α positivity between the cohorts.

The HIF-1α complex stabilisation and expression is central to cellular and systemic homeostatic responses to hypoxia. The activation of HIF-1α serves to improve survival under hypoxic conditions. Similarly, oncogene induced over expression and stabilisation of HIF-1α unrelated to tumour hypoxia is described (Swartz et al. 2015). HPV genes (E6 and E7) have also been linked to HIF-1α expression (Tang et al. 2007, Nakamura et al. 2009, Bodily et al. 2011, Rodolico et
Furthermore, in the context of hypoxia induced up regulation of HIF-1α, studies suggest endogenous hypoxia markers are influenced by the heterogeneity of tumour hypoxia in relation to differences in blood perfusion and oxygen diffusion. Therefore, studies on whole sections studies are better than TMAs for HIF-1α expression analysis.

In this study graded HIF-1α and CA-IX staining on tumour regions alone in whole tumour sections provided a unique opportunity to compare hypoxia induced HIF-1α expression in HPV positive and negative patients. The observation of significantly lower levels of HIF-1α in HPV positive patients may be related to the better prognosis observed in this group. HIF-1α up regulation is known to predict for a more aggressive treatment resistant phenotype (Le et al. 2004, Zhu et al. 2005). Conversely, there was no difference in CA-IX expression between HPV positive and negative patients. This may be related to a delay in HIF-1α induced transcription of CA-IX.

Evaluating the relationship between clinicopathological features and patients survival showed that increased tumour size was an adverse prognostic factor in both HPV positive and negative patients. There was no association between survival with AJCC stage and survival. Previously, AJCC was strongly linked to patient survival in OPSCC (Mamelle et al. 1994, Guerry et al. 1998, Layland et al. 2005). However, the changing prognostic significance of the AJCC system in OPSCC over time has been reported (Hong et al. 2013, Oguejiofor et al. 2013, Spector et al. 2014, Keane et al. 2015), particularly in HPV positive patients. Tumour size as reflected by T stage is consistently prognostic for patient outcome in HPV positive and negative groups, but some studies suggest HPV status is more important.

Lower expression of hypoxia markers (HIF-1α and CA-IX) showed a no significant association with improved outcomes in the combined analysis. This relationship was seen in both HPV positive and negative patients. The mechanism supporting the improved prognosis with lower HIF-1α and/or CA-IX expression can be extrapolated from the mechanisms and stimuli for hypoxic marker expression. Treatment resistance and an aggressive tumour phenotype are linked to the hypoxic response. A few have reported an opposite relationship with HIF-1α expression associated with a good prognosis (Fillies et al. 2005, dos Santos et al. 2012).
In a systematic review 40 studies across different treatment approaches and using a variety of hypoxia markers, Swartz et al reported that high expression of endogenous hypoxia markers was associated with a worse outcome in head and neck cancers (Swartz et al. 2015). Similarly, Jonathan et al reported a high CA-IX expression in patients in a phase II trial of accelerated radiotherapy with carbogen and nicotinamide (ARCON) was associated with improved survival (Jonathan et al. 2006). This might be related to the hypoxia modification component (carbogen and nicotinamide) which will improve tumour radiosensitivity. Furthermore, in a trial of hypoxia modification using nimorazole, Eriksen et al reported that CA-IX had no prognostic significance (Eriksen et al. 2007) in both placebo and treatment arms. In that study there was no stratification by HPV status, which might explain the poor prognostic ability of CA-IX.

In a study of 233 OPSCC patients, HIF-1α expression was associated with improved outcome in the HPV negative but not positive patients (Hong et al. 2013). In another study in OPSCC, Rahimi et al evaluated HIF-1α and CA-IX amongst other markers and observed no significant relationship with improved survival with both markers in a univariate analysis (Rahimi et al. 2012). In the study reported here there was a consistent association between CA-IX, HIF-1α and patient survival. Multivariate models in HPV positive and negative patient groups showed CA-IX but not HIF-1α was the most important predictor of survival.

This is the first study demonstrating the prognostic strength of CA-IX in a multivariate model in HPV positive and negative OPSCC. The use of a single biopsy might limit the finding as hypoxia is known to vary across the tumour. The result from this study further strengthens the importance of hypoxic markers in both HPV positive and negative tumours. In addition it does show the evolving paradigm of prognosis in OPSCC complementing known traditional factors. Last, clinical pathology reports and clinical trials need to include tumour hypoxia in prognostic groupings in both HPV positive and negative OPSCC.
7.0 Final Discussions

Differences in the epidemiology, aetiology and biology of oropharyngeal cancers present opportunities for individualised treatment options. Biomarker identification/selection in the context of translational research allows for evaluation of the successes of different treatment options. However, this is dependent on proper patient selection. This thesis began by describing a clinical test for HPV detection in OPSCC. It then validated a multiplex IHC method, which was used to demonstrate a variation of immune cell counts with increasing distances from an index section. Multiplex IHC evaluation of TIL density in tumour and stroma compartments of HPV positive and negative OPSCC showed higher infiltration in HPV positive tumours and a relationship between CD8+ T cell infiltration and patient survival in HPV positive patients. Expression of PD-L1 alone, although higher in HPV negative tumours, was not linked to patient outcome. A study evaluating biomarkers of hypoxia concluded this thesis. The overall aim of this body of work was to contribute knowledge to the rapidly evolving landscape of OPSCC by specifically describing prognostic factors operational in relation to HPV status.

7.1 HPV detection in OPSCC

The increasing incidence of HPV associated OPSCC and its association with improved clinical outcomes irrespective of treatment options inspired the development of trials studying less intensive treatment regimens. The rationale behind the de-escalation trials is to reduce the morbidity associated with standard therapy in younger patients with a better probability of survival (Mirghani et al. 2015). Consequently, misclassification of these patients could have dire consequences. Currently there are several registered de-escalation and therapeutic vaccination trials on clinicaltrials.gov (Table 7.1). HPV status is being assessed with p16 IHC alone or with HPV DNA detection by PCR or ISH. Issues with these methods as described in Chapters 1 and 2 might affect the outcomes of these studies or worse the outcome of patients in these studies.

It is pertinent to mention that individually or in combination these tests will demonstrate the broad prognostic importance of HPV in OPSCC. However, individualised patient risk with HPV misclassification and the attendant consequences must be at the fore-front of discussions of HPV detection methods and
not their performance as prognostic classifiers. The issue lies in misclassification of HPV negative patients as HPV positive rather than vice versa. Therefore, an overly sensitive detection test has an increased chance of such errors. In this study, p16 IHC had the highest sensitivity of the individual tests and a lower positive predictive value when compared to HPV mRNA detection. Although p16 is a sensitive marker for HPV detection it suffers from low specificity (Lewis 2012). Similarly, p16 might be up-regulated by other mechanisms unrelated to HPV (Perrone et al. 2011). The issues in sensitivity and specificity notwithstanding, p16 IHC is the most widely used assay for HPV detection in OPSCC (Chung et al. 2014). This is because it is relatively cheap and easier to perform and replicate in laboratories and clinics. Therefore, despite its shortcomings as a standalone test, it is continuously being used for HPV detection.

The popularity of p16 IHC, its higher sensitivity with low positive predictive value suggest that at best it should be used only as an initial screening test. Patients who are p16 positive should be subjected to a confirmatory test identifying and possibly typing transcriptionally active HPV. HPV mRNA detection as described and suggested in this thesis and by others (Smeets et al. 2007) is the “gold standard”. As it allows for precise HPV detection without the inconvenience of multiple testing. The HPV mRNA detection method described in Chapter 2 presents an opportunity for HPV typing with multiple analyte detection and correlations. Others have suggested the use of high throughput next-generation sequencing platforms. These methods are currently costly and not widely available, but are being evaluated for accuracy in detecting HPV in pre-cancerous (Yi et al. 2014) or cancerous (Chandrani et al. 2015, da Fonseca et al. 2015) tissue.

The drawback of a next-generation sequencing method is that in-depth analysis of transcriptomes without contextualisation to the tumour micro-environment provides no in-situ information. In contrast, the RNAscope platform allows for multiplex in-situ detection of mRNA in sections (Wang et al. 2012). Detection of high-risk HPV E6/E7 mRNA has been described (Wang et al. 2014). However, RNAscope does not permit quantification of mRNA levels and will be limited by the number of fluorophore combinations in a multiplex. At present the QuantiGene mRNA assay is relatively cheaper, easier to perform with a quick turnaround time. In addition it allows for HPV typing, mRNA quantification and potential to complex with other
analytes. Although the QuantiGene method does not allow for detection in-situ, future work could explore micro dissection of tumour areas. This will allow for the quantification of HPV mRNA and correlations with areas of immune activity and/or hypoxia. This test is the ideal confirmatory test in a two-step testing algorithm or as a single diagnostic test where one test is required.

Table 7.1 Ongoing trials evaluating de-escalation regimen in HPV+ve OPSCC

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Phase Number</th>
<th>Patients characteristics</th>
<th>Intervention</th>
<th>HPV detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01302834</td>
<td>III</td>
<td>706</td>
<td>RT (70 Gy) with weekly Cetuximab vs. Cisplatin</td>
<td>p16 IHC</td>
</tr>
<tr>
<td>RTOG 1016</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01874171</td>
<td>III</td>
<td>304</td>
<td>RT (70 Gy) with weekly Cetuximab or Cisplatin</td>
<td>p16 IHC</td>
</tr>
<tr>
<td>De Escalate HPV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01855451</td>
<td>III</td>
<td>200</td>
<td>RT (70 Gy) with Cetuximab vs. Cisplatin</td>
<td>p16 IHC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01663259</td>
<td>II</td>
<td>36</td>
<td>RT (70 Gy) with Cetuximab</td>
<td>p16 IHC + HPV DNA PCR</td>
</tr>
<tr>
<td></td>
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<td></td>
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</tbody>
</table>
| De-intensification of radiation and chemotherapy dose

| NCT01530997 | II           | 40                       | IMRT (54–60 Gy) with weekly Cisplatin | p16 IHC and/or HPV DNA PCR |
| NCT01088802 | II           | 60                       | IMRT, de-escalation (from 70 to 63 Gy and from 58.1 to 50.75 Gy, Cisplatin for first and last 3 weeks) | p16 IHC and/or HPV DNA PCR |
| NCT01891695 | I            | 45                       | Elective nodal dose de-escalation (39.6 Gy instead of 50 Gy) | p16 IHC |
|              |              |                          |              |               |
| Induction chemotherapy with lower radiation dose

| NCT01084083  | II           | 80                       | ICT (paclitaxel, cisplatin and cetuximab) followed by low (54 Gy) vs. standard dose IMRT with cetuximab | p16 IHC and/or HPV DNA PCR |
| ECOG 1308    |              |                          |              |               |
| NCT01706939  | III          | 365                      | ICT 56 Gy + cetuximab vs. 70 Gy + platin IMRT only, respectively, | p16 IHC + HPV DNA PCR |
|              |              |                          |              |               |
| Initial surgery

| NCT01932697  | II           | 40                       | Surgery followed by hyperfractionated IMRT docetaxel | p16 IHC |
|              |              |                          |              |               |
| NCT01898494 | II | 377 | Stage III-IVa (no T1–2 N1) | surgery with risk stratification: Low-risk no adjuvant therapy -Intermediate-risk randomized 50 vs. 60 Gy IMRT -High-risk patients IMRT (66 Gy) with cisplatin | p16 IHC |
| NCT01687413 | III | 496 | T1–4a with negative margins no extracapsular spread | Transoral surgery then randomization to IMRT (60 Gy, 30#) alone vs. with cisplatin | p16 IHC |
| NCT01493154 | I | 21 | Stage III-IV | HPV DNA vaccine (pNGVL-4a-CRT/E7 detox) by electroporation in combination with cyclophosphamide | HPV DNA ISH |
| NCT01598792 | I | 36 | HPV-16 +ve, p16 +ve OPSCC Patients in remission from disease | Recombinant Listeria Monocytogenes-based vaccine encoding HPV 16 antigens | p16 IHC + HPV DNA PCR |

RT – radiotherapy, IMRT – intensity modulated radiotherapy, ICT – induction chemotherapy. Adapted from (Mirghani et al. 2015)

7.2 The immune system, hypoxia and prognosis in OPSCC

The TNM staging classification is important but not always sufficient for prognostic classification in different cancer types. This is particularly evident in HPV positive OPSCC (Oguejiofor et al. 2013). The differential or similar responses of the same or different sized tumours respectively suggest the contribution of external influences (Galon et al. 2014), such as the immune system. The multiplex IHC method described and validated in Chapter 3 allows for characterisation, enumeration and contextualisation of TILs in the immune micro-environment. This thesis, showed that enumeration of TILs in sequential single sections is not an optimum method for evaluating TILs in sections. This might account for the differences reported in the literature. The data from this thesis supports the use of multiplex IHC methods in TIL studies. Standardisation is required across centres to allow for objective quantification of TILs in OPSCC and possibly other cancers.

The OPSCC patients included in this thesis underwent treatment with radiotherapy. Furthermore, the local effects of the immune micro-environment and
hypoxia in potentiating and limiting the effects of radiotherapy respectively are the central message in this thesis (Chapter 4 and 6). Available evidence suggests that hypoxia pathways are involved in regulating the function of the immune system in the micro-environment (Kumar and Gabrilovich 2014). Similarly, previous assumptions on the immunosuppressive effects of radiation are currently being revisited in studies investigating the local immune reaction to radiation (Formenti and Demaria 2013). The development of cancers and their recognition by initially innate and subsequently adaptive immune mechanisms is described (Dunn et al. 2004, Schreiber et al. 2011). This allows for either control or positive selection of an immune resistant phenotype, which precedes cancer spread (Dunn et al. 2002, Dunn et al. 2006). It is thought that radiation “resets” the milieu by exposing neo-antigens and permitting their recognition by the immune system (Galluzzi et al. 2007, Ma et al. 2010). Therefore, tumours with primed immune responses (evident by increased TILs) stand a better chance of being controlled following radiotherapy. This theory underlies the observation in this thesis and of others of favourable outcomes in patients with higher immune cell infiltration (Galon et al. 2014).

The mechanism of action of radiation induced immune activations involves the activation of both innate and adaptive responses. This is dependent on radiation induced immunogenic cell death mediated by pro-inflammatory cytokines (CXCL9, CXCL10, CXCL16, IL-1b, TNF α, Type 1 and 2 IFN) (Lugade et al. 2008, Matsumura et al. 2008) that promote the recruitment of CD8, CD4 and macrophages (Burnette et al. 2011, Lim et al. 2014). Also observed is up-regulation of MHC class 1 molecules (Garnett et al. 2004), death receptors (Ifeadi and Garnett-Benson 2012), co-stimulatory molecules (Derer et al. 2015) and danger associated patterns (Kroemer et al. 2013). The immune activation on a tumour following radiotherapy also promotes immune activity at non irradiated sites in so called “abscopal effects”, which may further potentiate the effects of radiotherapy (Reynders et al. 2015). However, the successful induction of tumour immunogenicity is dependent on patient genetic polymorphisms of immune cell receptors, intrinsic tumour properties and expression of immunosuppressive ligands in the micro-environment (Formenti and Demaria 2013).

Targeting immunosuppressive pathways has shown impressive results in early clinical trials (Hodi et al. 2010, Brahmer et al. 2012). As radiotherapy augments the
immune microenvironment, the synergestic effects of radiation and therapeutic blockade of PD-L1 pathways have been investigated (Deng et al. 2014, Twyman-Saint Victor et al. 2015). In mouse models, Deng et al observed an increased expression of the PD1/PD-L1 axis following radiotherapy and reduction of myeloid derived suppressor cell density (known to suppress T cell functions) with combination therapy (Deng et al. 2014). Similarly, others observed increased PD-L1 expression following combination therapy (radiation and anti-CTLA4) which predicted resistance in humans and mouse models (Twyman-Saint Victor et al. 2015). These observations and data from clinical trials demonstrate the potential for radiation and immune combinations. However, issues with patient selection, timing of immunotherapy and prognostic and/or predictive roles of these markers remain unresolved. Dovedi et al observed that concomitant administration of radiotherapy and anti-PD-L1 mAb led to better survival in mouse models (Dovedi et al. 2014). Therefore, as PD-L1 expression is increased by radiotherapy, pre-treatment observations are insufficient for prognostic classification (as shown in Chapter 5). The data from this study did not demonstrate any prognostic role for the expression of PD1/PD-L1 alone, but only for macrophage expression of PD-L1 in HPV negative tumours. Post radiotherapy observations of the immune microenvironment are required to investigate changes and optimise immunotherapy in response to dynamic fluctuations of the immune system. In the context of HPV positive and negative OPSCC the divergent molecular properties of the tumours may account for their differential responses to radiation and immune factors. Future work by the group will investigate the similarities and/or differences with cervical cancer with specific reference to TIL density.

These observations are further complicated by the effects of tumour hypoxia on immune activity. Available evidence suggest that HIF is involved in the regulation of immune cells in the tumour micro-environment. Lukashev et al observed that deletion of the I.1 isofrom of HIF-1α (responsible for <30% of total HIF-1α mRNA) was sufficient to enhance T cell receptor triggered cytokine secretion, suggesting that HIF-1α is a negative regulator of T cell function (Lukashev et al. 2006). In addition, NANOG mediated hypoxic cell resistance to cytotoxic T cell activity has been observed (Lukashev et al. 2007, Hasmim et al. 2011). Also, in human and murine cell lines, Barsoum et al reported PD-L1 upregulation dependent on HIF-1α levels;
increased PD-L1 expression in-vivo was linked to resistance to cytotoxic T cell mediated lysis (Barsoum et al. 2014). Furthermore, HIF-1α induced upregulation of PD-L1 on macrophages, dendritic cells and tumour cells has been observed in tumour-bearing mice (Noman et al. 2014). In this study, HPV negative tumours had higher expression of both HIF-1α and PD-L1 expression, and HPV positive tumours as a group had lower HIF-1α expression and higher TILs. Pertinently, HPV positive and negative patients had similar responses to hypoxia. The observations in this study will be further validated in two patient cohort from sites in Birmingham and Australia. However, trials of hypoxia modification should be conducted in both patient groups. This is contrary to the ongoing European Organisation for Research and Treatment of Cancer (EORTC) trial evaluating nimorazole in HPV negative patients (ClinicalTrials.gov identifier NCT01880359), which uses p16 IHC alone for HPV classification.

In summary, studies evaluating co-localisation of HIF-1α, immune cell densities and PD-L1 expression are needed. Pre- and post-treatment observations will allow for characterisation of the relationships between radiation, immune system and hypoxia. This will provide evidence for the evaluation of radiotherapy with simultaneous blockade of PD-L1 and targeting of hypoxic cells/inhibition of HIF-1α in a clinical trial. Presently, the group is investigating the relationship between TIL density, PD1/PD-L1 and hypoxia marker expression in sarcoma tissue. It is important that this approach is extended to other tissue types to observe for similarities and/or differences in different tumour types. It is becoming evident that, in the future, complementary therapies individualised to unique patient characteristics will become the standard of care in cancer therapy.

In conclusion the work carried out in this thesis showed that: 1) different HPV detection methods are associated with differences in patient clinical characteristics and outcome; 2) multiplex IHC avoids the issues encountered with single IHC particularly in the evaluation of immune cell activity the immune micro-environment; 3) using the multiplex IHC there are observable differences in the density of TILs between HPV positive and negative patients, which is linked to patient survival in HPV positive patients; 4) in HPV negative patients an inflammatory phenotype characterised by increased macrophage expression of PD-L1 and increased presence of CD8+ T cells identifies patients likely to respond
favourably to radiotherapy; 5) hypoxia response is associated with a poor prognosis in both HPV positive and negative OPSCC. Therefore, this thesis concludes that: a) HPV detection in the context of clinical trials of de-escalation in head and neck cancer need to be revisited; b) TIL and hypoxia reporting should be included in patient prognostic classification schema; c) pre-treatment expression of PD-L1 is insufficient to identify patients likely to respond to radiotherapy; and d) trials of hypoxia modification in head and neck cancer should include both HPV positive and negative patients.

7.3 Synopsis and future directions

Evidence for the prognostic importance of HPV status in OPSCC has been described above. Therefore, the current mono-phasic treatment approach in OPSCC is unsustainable. Data from ongoing treatment de-escalation trials are soon expected and would provide a guide for future treatment stratification based on HPV status. In the context of treatment de-escalation, proper HPV detection is required and requisite for the development of any treatment paradigm in OPSCC. Overtime HPV mRNA detection will become the standard for HPV classification as it identifies transcriptionally active HPV cancers as opposed to current detection methods. However as shown above HPV status alone is insufficient for prognostic stratification as there are other factors operating within these broad prognostic oversimplifications. Identification of these factors within and outside HPV classification (as described in this thesis) are important to allow for prognostic stratification and the development of selective therapy with associated less morbidity.

Targeted therapy approaches are currently in use in HNSCC, Cetuximab a chimeric immunoglobulin mAB targeting the external domain of EGFR has been widely reported on (Machiels et al. 2014, Adams et al 2014, Bonilla-Velez et al. 2014). The interruption of uncontrolled growth factor kinetics by extracellular or intracellular inhibition although important is insufficient to completely interrupt oncogenesis in HPV positive or negative OPSCC. As suggested in this thesis a two step model involving: 1) individualised and 2) combination therapy approaches provides the best approach for the future of therapy in OPSCC and in other cancers. Individualised therapy provided to patients as stratified by multiparameter prognostic
models developed using intrinsic (mutation profile) and extrinsic (immune characteristics, hypoxia and others) factors. Appropriate treatment combinations (dependent on prognostic factors as above) should be offered dependent on prognostic stratification at diagnosis and continuously modulated with development of treatment resistance and disease recurrence.

Data from this thesis provides evidence for the prognostic role of immune system and hypoxia in OPSCC. Specifically, it demonstrates the influence of cytotoxic immune system and inflammation in HPV positive and negative OPSCC respectively. The future of therapy in OPSCC will be dependent on the successes of prevention strategies and the development of novel less toxic treatment approaches. Prevention strategies (smoking/alcohol cessation and use of prophylactic HPV vaccines) will lead to the incidence reduction and long term modulation of epidemiology in OPSCC. However, for patients with already established HPV infection and or cancers, immune modulatory treatment provides the optimum approach (Lin et al. 2010, Ma et al. 2012). Viral proteins (E6 and E7) are ideal targets as they are universally expressed and entirely foreign. Adoptive T cell transfer (ClinicalTrials.gov identifiers; NCT02379520 and NCT02280811) therapeutic vaccination (ClinicalTrials.gov identifiers; NCT01598752, NCT02002182, NCT01493154) directed against these viral proteins are currently under investigation in clinical trials in HPV positive OPSCC. However, the success of immune-modulatory approaches in OPSCC and in other cancers is limited by: 1) genetic determinants of immune response and 2) concurrent iatrogenic immunosuppression in most cancer treatment protocols. Conversely, approaches using antibodies labelled with radiation in so called “radioimmunotherapy” (Wong et al. 2006) to selectively target E6 and E7 expressing cancer cells without the active participation of the host immune system will be the optimum approach. In HPV negative OPSCC better targets are required.

In conclusion this thesis has contributed to the further characterisation of the prognostic factors in oropharyngeal cancers. To provide a model for individualised treatment approach further moving cancer therapeutics into the realm of personalised medicine.
8.0 References


the palatine tonsil characterized by distinct etiology, molecular features and outcome." 


causal association between human papillomavirus and a subset of head and neck cancers." J Natl Cancer Inst 92(9): 709-720.


8.0 Appendices

Appendix 1: List of publications and book chapter contributions produced during the PhD


Appendix 2: First author publications produced during the PhD

1. The prognostic significance of the biomarker p16 in oropharyngeal squamous cell carcinoma

Clinical Oncology 25 (2013) 630–638

Original Article

The Prognostic Significance of the Biomarker p16 in Oropharyngeal Squamous Cell Carcinoma

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Abstract

Aims: There is an increasing incidence of human papillomaviruses (HPV)-positive oropharyngeal squamous cell cancers (OPSCC) mostly associated with favourable outcomes. p16 immunohistochemistry is a surrogate marker for HPV positivity in OPSCC. The prognostic strength of p16 over traditional prognostic factors is not fully characterised. In this study, we evaluated the clinical and demographic differences between p16-positive and -negative OPSCC and characterised its prognostic strength versus traditional prognostic factors.

Materials and methods: Formalin-fixed, paraffin-embedded blocks and clinical information from 217 OPSCC patients, treated with radiotherapy (alone or in combination with other therapies) between 2000 and 2009 were collected retrospectively. Immunohistochemistry for p16 protein was carried out; cancer-specific survival (CSS), recurrence-free survival (RFS) and loco-regional control (LRC) were calculated for both univariate and multivariate analyses.

Results: Ninety-two per cent of the OPSCC originated from tonsillar and tongue base sites, 61% were p16 positive. Patients with p16-positive OPSCC were younger ($P < 0.0001$), with lower alcohol ($P = 0.0002$) and tobacco ($P = 0.0001$) exposure. The tumours were less differentiated ($P = 0.0069$), had lower T stage ($P = 0.0027$), higher nodal status ($P = 0.014$) and higher American Joint Committee on Cancer (AJCC) prognostic group ($P = 0.0038$). AJCC prognostic group was significant for RFS ($P = 0.0006$) and CSS ($P = 0.018$) in patients with p16-negative OPSCC, but not those with p16-positive tumours ($P = 0.30$ and 0.54). Other significant factors for CSS and RFS in univariate analysis were: pretreatment haemoglobin ($P = 0.0001$ and $P = 0.0001$); chemoradiotherapy ($P = 0.005$ and 0.035) and p16 status ($P = 0.0001$ and 0.0001). In multivariate analysis, p16 positivity was the strongest independent prognostic variable for both CSS, RFS and LRC ($P < 0.0001$, hazard ratio 4.15; 95% confidence interval 2.43–7.08; $P < 0.0001$, hazard ratio 6.15; 95% confidence interval 3.57–10.61) and ($P < 0.0001$, hazard ratio 3.74; confidence interval 1.76–7.95).

Conclusions: This study shows that p16 is the single most important prognostic variable in OPSCC, surpassing traditional prognostic factors for both CSS and RFS. Furthermore, disease stage has no prognostic significance in p16-positive patients, highlighting the need for routine p16 assessment in OPSCC.

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Keywords: Chemoradiotherapy; head and neck cancer; human papillomavirus (HPV); oropharyngeal cancer; p16; prognosis

Introduction

Human papillomaviruses (HPV) are non-enveloped double-stranded DNA viruses commonly associated with cervical cancers [1]. High-risk types are also linked with cancers of the oropharynx (oropharyngeal) squamous cell cancers; OPSCC) affecting primarily tonsillar and tongue base sites [2]. Emerging trends suggest an increase in the prevalence of HPV-associated OPSCC mostly in young people with lower exposure to tobacco and alcohol [3–7]. Similarly, reports suggest that these cancers are distinct from other head and neck cancers with differing biology, morphology and prognosis [8]. HPV-positive OPSCC tend to be poorly differentiated with basaloid morphology, presenting with a lower T stage and higher nodal status [2], but paradoxically better prognosis [9–11]. Presently, there is considerable interest in detecting these cancers for risk stratification, epidemiological studies, de-intensification of current
treatment regimens and for possible initiation of viral-targeted therapy (vaccination and/or antiviral agents). Currently, available methods detect DNA, RNA or surrogate markers of HPV infection. HPV DNA is detected using polymerase chain reaction and/or in situ hybridisation, but DNA-based analysis is imperfect [12]. p16 immunohistochemistry (IHC) is used as an alternative and/or complementary test, the p16 protein is encoded by CDKN2A, a tumour suppressor gene located on chromosome 9p21 that is frequently lost in head and neck squamous cell cancer [13]. p16 is involved in maintaining the integrity of the G1/S cell cycle check point through binding to cyclin-dependent kinases, which are involved in the release of the E2F transcription factor by the phosphorylation of retinoblastoma protein. Inactivation of retinoblastoma by the HPV E7 leads to the subsequent up-regulation of the p16. As p16 is usually lost early in HPV-unrelated OPSCC [14,15], tests detecting p16 are used as a surrogate marker of HPV-induced oncogenesis.

In most studies, p16 IHC alone is a strong prognostic factor consistently linked to differences in overall, recurrence-free (RFS) and disease-specific survival that is independent of treatment type [10]. Presently it is not known if all p16-positive OPSCCs share similar outcomes [16–18], with considerable effort being made to identify tumours with discordant HPV/p16 results. Similarly, treatment options in OPSCC have generated considerable interest. In general, OPSCC is treated similarly despite available evidence suggesting biological, morphological and survival differences. This has led to de-escalation trials for HPV-positive cases that aim to improve survival while limiting morbidity and increasingly to surgical approaches for fitter patients with HPV-negative disease.

In this study we evaluated the differences in the clinicodemographic, survival and treatment options between p16-positive and -negative OPSCC in a large cohort of patients. The prognostic strength of p16 was compared with other traditional prognostic factors in OPSCC and the importance of prognostic stage was compared in p16-positive and -negative patients.

Materials and Methods

Patients

The 217 patients in this study were treated at The Christie NHS Foundation Trust Hospital. The inclusion criteria were: histologically confirmed OPSCC; treatment between January 2000 and December 2010; and the use of radiotherapy as one or the only modality of treatment. Patients treated with palliative intent were excluded. Patient information was collected in two cohorts 1999–2004 and 2005–2010. Treatment involved: radiotherapy alone (intensity-modulated radiotherapy or conventional radiotherapy [60–66 Gy over 6–6.6 weeks or 55 Gy in 4 weeks]), primary neck surgery and radiotherapy, or chemoradiotherapy (induction and/or concurrent chemoradiotherapy). Induction and concurrent chemotherapy comprised: docetaxel, cisplatin, 5-fluorouracil and cisplatin or carboplatin respectively. Ethical approval for the study was obtained from the local research ethics committee (reference number 03/TC/076; Christie reference number 03_RADIO_22).

Immunohistochemistry

Formalin-fixed, paraffin-embedded blocks were obtained from referring hospitals. Sections were cut and stained with haematoxylin and eosin. Blocks with confirmed tumour presence were selected for IHC, which was carried out using the CellNec histology kit (MFM Laboratories, Heidelberg, Germany) as per manufacturer’s instructions. Briefly, 4 μm sections were deparaffinised and rehydrated. This was followed by epitope retrieval in a water bath using epitope retrieval solution (CellNec). The solution and slides were then heated to 97 °C for 10 min and cooled at room temperature for 20 min. The staining was carried out on a Biogenex 16000 autostainer as per the manufacturer’s protocol. The slides were then counterstained with Gill’s haematoxylin and cover-slipped. Controls were included with each batch, and were HPV-positive and -negative samples. No batch-to-batch variation was encountered between controls.

Scoring

The stained slides were scanned using the Leica SCN 400. p16 expression was scored as positive if there was a strong and diffuse brown staining of the nucleus and cytoplasm in ≥70% of the tumour specimen [19]. The slides were scored by a single scorer with high (R² = 0.91) intra-scorer reproducibility.

Statistical Analysis

Statistical analyses were carried out using SPSS version 16.0. Actuarial calculations of locoregional control (LRC), RFS and cancer-specific survival (CSS) were obtained using the Kaplan–Meier method. CSS was used rather than overall survival to avoid any bias in comparing younger and healthier HPV-positive patients (less likely to die of secondary causes) with HPV-negative patients. Univariate analysis was compared using the log-rank (Mantel-Cox) method. The Cox proportional hazard model was used for multivariate analysis. The time to primary re-occurrence was defined from the start of treatment with radiotherapy to reported recurrence.

Results

There were no differences in survival in both cohorts 1999–2004 and 2005–2010 (Supplementary Figure 1). The 217 patients included in the study had a median age at presentation of 58.5 years. As expected, most of the tumours (92%) originated from tonsil and tongue base sites. Most of the tumours had high clinical stage American Joint
Committee on Cancer; AJCC) disease (64% stage IV) and most were moderately differentiated (53%). The prevalence of p16-positive tumours in this cohort was 61% (Table 1).

**p16 Status and Clinical Characteristics**

Table 2 compares the clinical characteristics of p16-positive and -negative tumours. p16-positive tumours occurred more frequently in younger patients, with a mean age at treatment of 56 years (95% confidence interval 55–57) versus 64 years (95% confidence interval 63–65) years (P < 0.0001) (Figure 1). In comparison, p16-negative tumours, p16-positive tumours were also associated with non-smokers, low alcohol use, low T stage, high N stage, high AJCC stage and poor differentiation. p16-positive patients more likely to have received chemoradiotherapy (P = 0.0072); there was no difference in gender (P = 0.51) or tumour site (P = 0.41) when the clinical characteristics of p16-positive and -negative tumours were compared.

**Univariate Analysis**

Figure 2 shows Kaplan–Meier curves stratified by p16 status (Figure 2A, B) and chemoradiotherapy (Figure 2C, D). On univariate analysis, smoking, pretreatment haemoglobin, T stage, nodal status, AJCC stage, differentiation, chemoradiotherapy and p16 status were significantly associated with LRC, RFS and CSS (P < 0.001). Age (P = 0.004) was significantly associated with CSS alone (Table 3). We also looked at the interaction between p16-positive/negative patients and the use of chemoradiotherapy (Figure 2E, F). We saw a trend for better survival among the p16 patients, which was less evident in the p16-negative patients. Given that p16-positive patients present with a higher grouping, we evaluated the influence of prognostic group (AJCC prognostic group) on survival and observed a significant relationship in the p16-negative patients (Figure 3A, B). This relationship was not seen in the p16-positive patients (Figure 3C, D).

**Multivariate Analysis**

Factors associated with LRC, RFS and CSS on univariate analysis (smoking, pretreatment haemoglobin, T stage, nodal status, AJCC stage, grade and p16 status) were included in the Cox proportional hazards model. P16 was significant on multivariate analysis for all end points (Table 4). Tumour grade was significant for both RFS (P = 0.004) and CSS (P = 0.001). Pretreatment haemoglobin (P < 0.001) was associated with RFS. T stage was a significant independent prognostic factor for LRC (P = 0.001) and CSS (P = 0.001). p16 was the strongest independent prognostic variable identified in this study for LRC (hazard ratio = 3.74; 95% confidence interval 1.76–7.95; P = 0.001), RFS (hazard ratio = 6.15; 95% confidence interval 3.57–10.61; P < 0.0001) and CSS (hazard ratio 4.15; 95% confidence interval 2.43–7.08; P < 0.0001).

**Discussion**

The prevalence of OPSCC arising from tonsil and tongue base sites in young non-smoking adults is reported to be on the rise globally [3,4] and in the UK [7]. To our knowledge, this is the first study in the UK to study a large series of patients over a 10-year period, observing differences in clinicodemographic, survival and prognostic features between p16-positive and -negative tumours. The prevalence rate from this study was 61%. However, as this is a radiotherapy-treated cohort from a single site, the true prevalence in the UK remains largely unknown.

In this study, we observed the consistent finding of differing clinicodemographic characteristics between p16-
positive and -negative tumours, p16-positive tumours occurred in younger patients, a finding that has been consistently reported by others [8, 20, 21]. The median age of 56 years (p16 positive) reported in this study is similar to the finding from the MACH-ANCA trial [21]. Similarly, when lifestyle choices were examined, patients with p16-positive OPSCC were less likely to be smokers and/or drinkers, a feature that has been previously reported [8, 20] and is one of the epidemiological characteristics underlying the aetiological association between HPV positive versus negative OPSCC. There were pathological differences between p16-positive and -negative tumours, with positive tumours presenting with a lower T stage and a higher nodal status and as such a higher AJCC stage. This feature has been reported by others [2, 22, 23]. In the univariate analysis, smoking status was significantly associated with LRC, RFS and CSS, which has been reported previously [24]. In this study, p16 positivity showed a highly significant association with RFS and CSS in univariate analysis. Moreover, it was an independent prognostic factor in this cohort after multivariate analysis, a feature that has been reported by others [25, 26]. p16 has been shown to be a strong prognostic biomarker after radiotherapy alone [26], chemoradiotherapy [24, 27 – 29], primary surgery with or without adjuvant radiotherapy [17, 30, 31]. In this study, 5 year CSS and RFS rates for all patients were 56% and 58%, respectively, which are similar to the reports from a meta-analysis [10]. Stratifying patients based on p16 status, improved 5 year survival rates from 38% to 75% for CSS and 28% to 78% for RFS, similar improvements have been observed by others [29, 24, 29, 32]. In this study we also evaluated the effect of chemoradiotherapy on survival and observed an improvement from 51% to 76% and 49% to 75% for CSS and RFS, respectively. This may have been influenced by the greater number of p16-positive patients in the chemoradiotherapy arm, as p16 patients are younger with better survival rates irrespective of treatment type. To determine whether chemotherapy benefited both p16-positive and -negative groups equally, we stratified the patients and observed a trend for improved survival in the p16-positive group, which was less obvious in the p16-negative group. Although this is a novel finding, the observations were not statistically significant and, due to the small numbers in each arm, the finding must be interpreted with caution. However, it would be worthwhile repeating this analysis in a larger cohort. The retrospective nature of data collection could limit this study, as could the heterogeneity of radiotherapy treatment over a 10 year period. That said, we present a relatively large cohort of OPSCC patients who underwent radiotherapy with curative intent.

What is interesting from the current study is that the prognostic strength of p16 surpasses the traditional prognostic markers such as TNM classification and prognostic group in the multivariate analysis; this again has been reported previously [9, 25]. In their study, Fischer et al. [25] evaluated prognosis in 392 patients using p16 IHC. Our larger cohort of 217 patients lends further strength to this observation. Given the paradox of p16-positive tumours presenting with higher stage and grade, but with better prognosis, we also explored the role of current clinical staging in p16-positive and -negative groups. Remarkably, the well-reported prognostic association of AJCC stage in OPSCC [33] was clearly shown in p16-negative tumours.
Fig 2. Kaplan–Meier curves of: (A) and (B) comparing 5 year cancer-specific survival (CSS) and recurrence-free survival (RFS) in p16-positive and -negative patients, respectively; (C) and (D) comparing 5 year CSS and RFS in patients treated with or without chemoradiotherapy, respectively; (E) and (F) comparing CSS and RFS in p16-positive and -negative patients, respectively, treated with chemoradiotherapy.

(P < 0.05), but was not significant in the p16-positive tumours (i.e. 61% of cases). Taken together, this shows not only that p16 is the most significant independent prognostic factor in this cohort, but also that the current prognostic classifications has no benefit in p16-positive tumours.

There is currently considerable discussion over the best way to classify HPV-positive and -negative tumours using the myriad of techniques available and p16 is not without faults. There are patients who are p16 positive, yet HPV DNA polymerase chain reaction and/or HPV DNA in situ hybridisation negative [16,38]. The use of p16 alone could lead to an overestimation of the true prevalence of HPV-positive OPSCC. Our intention was not to report on the prevalence of HPV, but rather to observe the differences in the clinico-demographics, tumour morphology and survival patterns between p16-positive and -negative tumours. Significantly larger studies are required to address whether...
Table 3
The univariate log-rank analysis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Locoregional control (Hazard ratio 95% confidence interval)</th>
<th>P value</th>
<th>Recurrence-free survival (Hazard ratio 95% confidence interval)</th>
<th>P value</th>
<th>Cancer-specific survival (Hazard ratio 95% confidence interval)</th>
<th>P value</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>Female</td>
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<td></td>
<td>1.94 (0.68–2.98)</td>
<td>0.50</td>
<td>1.48 (0.78–2.05)</td>
<td>0.22</td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
<td></td>
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<tr>
<td>&lt;50</td>
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<tr>
<td>50–60</td>
<td>0.85 (0.58–1.12)</td>
<td>0.9</td>
<td>1.49 (1.057–2.062)</td>
<td>1.68</td>
<td>1.19 (0.92–2.43)</td>
<td>0.004</td>
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<td>&gt;60</td>
<td>1.74 (1.25–1.84)</td>
<td>1</td>
<td>0.83 (0.46–1.25)</td>
<td>0.15</td>
<td>0.98 (0.54–1.32)</td>
<td>0.004</td>
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<tr>
<td>Low–medium</td>
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<td>1.25 (0.65–1.9)</td>
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<td>Heavy</td>
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<td>2.38 (1.12–4.93)</td>
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<tr>
<td>Ex</td>
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<td>0.014</td>
<td>0.70 (0.34–1.41)</td>
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<td>0.24 (0.08–0.69)</td>
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<tr>
<td>≤14</td>
<td>0.24 (0.69–0.86)</td>
<td>0.001</td>
<td>0.66 (0.30–1.44)</td>
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<td>0.33–1.68</td>
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<td>&gt;14</td>
<td>0.45 (0.19–1.06)</td>
<td>0.91</td>
<td>0.48 (0.18–1.70)</td>
<td>&lt;0.0001</td>
<td>0.96 (0.49–1.88)</td>
<td>&lt;0.0001</td>
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<td>T stage</td>
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<td>2</td>
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<td>4.15 (1.31–13.11)</td>
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<td>3.73 (1.18–11.78)</td>
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<td>2.29 (0.65–8.06)</td>
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<td>2.46 (0.76–7.94)</td>
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<td>0.39–5.49</td>
<td>0.003</td>
</tr>
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<td>4</td>
<td>2.29 (0.65–8.06)</td>
<td>0.38</td>
<td>2.29 (0.76–7.94)</td>
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<td>0.39–5.49</td>
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<tr>
<td>Nodal status</td>
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<tr>
<td>0</td>
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<tr>
<td>1</td>
<td>0.39 (0.07–2.14)</td>
<td>0.03</td>
<td>1.03 (0.33–3.39)</td>
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<td>0.21–2.33</td>
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</tr>
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<td>2</td>
<td>1.39 (0.47–4.09)</td>
<td>1.67</td>
<td>1.66–4.27</td>
<td>1.47</td>
<td>0.39–5.49</td>
<td>0.003</td>
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<tr>
<td>3</td>
<td>0.89 (0.08–8.03)</td>
<td>0.026</td>
<td>4.15 (1.31–13.11)</td>
<td>0.007</td>
<td>3.73 (1.18–11.78)</td>
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</tr>
<tr>
<td>AJCC stage</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.14 (0.03–7.44)</td>
<td>1.30</td>
<td>1.30 (0.31–5.46)</td>
<td>1.47</td>
<td>0.39–5.49</td>
<td>0.003</td>
</tr>
<tr>
<td>4a</td>
<td>1.13 (0.25–5.12)</td>
<td>1.40</td>
<td>1.40 (0.39–5.42)</td>
<td>1.18</td>
<td>0.34–4.09</td>
<td>0.003</td>
</tr>
<tr>
<td>4b</td>
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<td>2.84</td>
<td>2.46 (0.76–7.94)</td>
<td>2.31</td>
<td>0.62–6.48</td>
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<tr>
<td>4bc</td>
<td>3.78 (0.76–18.86)</td>
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<td>6.13 (1.69–23.7)</td>
<td>0.008</td>
<td>5.79 (1.78–16.86)</td>
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<tr>
<td>Tumour differentiation</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Well</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>1.41 (0.56–3.2)</td>
<td>1.41</td>
<td>2.09 (0.97–4.02)</td>
<td>2.61</td>
<td>1.10–6.19</td>
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<tr>
<td>Poor</td>
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<td>0.49</td>
<td>1.73 (0.78–3.83)</td>
<td>0.019</td>
<td>2.02 (0.82–4.96)</td>
<td>0.015</td>
</tr>
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<td>Chemoradiotherapy</td>
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<td>1</td>
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</tr>
<tr>
<td>No</td>
<td>1.30 (0.56–1.8)</td>
<td>0.45</td>
<td>1.97 (1.22–3.18)</td>
<td>0.005</td>
<td>1.78 (1.29–2.89)</td>
<td>0.03</td>
</tr>
<tr>
<td>p16 status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>5.01 (2.44–10.3)</td>
<td>&lt;0.001</td>
<td>6.07 (3.52–10.46)</td>
<td>&lt;0.001</td>
<td>4.60 (2.79–7.60)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

p16 positivity is a phenotype independent of HPV. However, given the aetiological link between HPV and p16, this seems unlikely. These discrepancies could equally arise through technical rather than through phenotypic differences. This is an area of debate and more importantly it is unclear whether tumours that are p16 positive but HPV negative share the same prognosis as p16-positive/HPV-positive patients [17,18]. Due to the frequency of discrepant cases, this would not be apparent in survival analysis, particularly in small cohorts. This knowledge is essential for the appropriate stratification of patients in prospective studies and larger, well-powered studies are needed. Retrospectively, however, p16 performs better than stage in terms of prognostic- tion. Presently, the National Comprehensive Cancer Network guidelines[14] recommend p16 IHC as a prognostic test. The British Association of Otorhinolaryngology on the other hand advises that in ‘routine clinical practice HPV testing is desirable but not mandatory’.
[35]. Given the overwhelming evidence that p16-positive patients consistently do better, regardless of treatment modality, including this cohort, all pathology departments should include p16 IHC in routine pathological assessment of OPSCC, as a simple, sensitive and robust initial triage of cases. We do not suggest limiting prognostication solely on the basis of p16 staining, rather we advocate its inclusion in any prognostic stratification, as available evidence suggest

Table 4
The multivariate Cox-regression analysis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Locoregional control Hazard ratio (95% confidence interval)</th>
<th>P-value</th>
<th>Rank</th>
<th>Recurrence-free survival Hazard ratio (95% confidence interval)</th>
<th>P-value</th>
<th>Rank</th>
<th>Cancer-specific survival Hazard ratio (95% confidence interval)</th>
<th>P-value</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour differentiation</td>
<td>Well 1: 2.03 (0.93–4.46)</td>
<td>0.046</td>
<td>2</td>
<td>Moderate 1: 2.01 (0.88–4.56)</td>
<td>0.004</td>
<td>1</td>
<td>Poor 1: 13.23 (1.75–99.55)</td>
<td>0.01</td>
<td>3</td>
</tr>
<tr>
<td>T stage</td>
<td>1 1: 2</td>
<td></td>
<td></td>
<td>2 1: 1.38 (0.28–6.57)</td>
<td>0.66</td>
<td></td>
<td>3 1: 2.28 (0.41–12.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment haemoglobin</td>
<td>≤12 1</td>
<td></td>
<td></td>
<td>&gt;14 1</td>
<td>0.14 (0.06–0.33)</td>
<td>0.001</td>
<td></td>
<td>1</td>
<td>0.37 (0.19–0.73)</td>
</tr>
<tr>
<td>p16</td>
<td>Positive 1</td>
<td>0.001</td>
<td>1</td>
<td>Negative 1</td>
<td>6.15 (3.57–10.61)</td>
<td>&lt;0.0001</td>
<td>1</td>
<td>4.15 (2.43–7.08)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NS, non-significant
it is the most important marker in survival of OPSCC. It is unclear what could be responsible for the differences in survival between p16-positive and -negative patients. One difference is the persistence of the pRB and p53 pathways in HPV-positive OPSCC and the loss in HPV-negative OPSCC [36–39], which may account for a better response to treatment strategies [16,40]. Furthermore, Stransky et al. [41] report that the mutation rate of HPV-positive tumours is half the rate of HPV-negative tumours [41]. The higher mutation rate in the HPV-negative tumours predisposes them to recurrence and resistance to therapy.

In conclusion, our study shows that p16 expression is the most significant prognostic factor in OPSCC after radiotherapy. We also show that AJCC stage has no prognostic significance in p16-positive patients and as such p16 IHC should be included in patient risk stratification.

Acknowledgement

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clon.2013.07.003.

References

[27] Takeh C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck...


2. Stromal infiltration of CD8 T cells is associated with improved clinical outcome in HPV-Positive oropharyngeal squamous carcinoma

Stromal infiltration of CD8 T cells is associated with improved clinical outcome in HPV-positive oropharyngeal squamous carcinoma

K Oguejiofor1,2, J Hall3, C Slater4, G Betts5, G Hall6, N Slevin7, S Dovedi6, P L Stern7 and C M L West5,1,2

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Background: Patients with human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) have a better prognosis than those with HPV-negative tumours. There is interest in de-escalating their treatment but strategies are needed for risk stratification to identify subsets with a poor prognosis. This study investigated tumour-infiltrating lymphocytes (TILs) in relation to HPV tumour status and patient survival.

Methods: Biopsies from 216 patients diagnosed with OPSCC between 2002 and 2011, who underwent chemo/radiotherapy were analysed for HPV by PCR, in-situ hybridisation and p16 immunohistochemistry (IHC). One hundred and thirty-nine samples with concordant HPV detection were analysed for CD3, CD4, CD8 and FoxP3 expression in tumour and stromal regions using multiplexIHC and multispectral image analysis. Labelling of smooth muscle actin (SMA) identified activated stroma.

Results: Human papillomavirus-positive compared with HPV-negative OPSCC had higher infiltration in both tumour and stromal areas of CD4 and CD8 T cells but not FoxP3 T regulatory cells. Only CD3+CD8+ stromal and not tumour area infiltration was associated with increased survival (P=0.02). There was significantly higher SMA expression in HPV-positive compared with -negative tumours, which did not correlate with survival.

Conclusions: Studies of TILs for risk stratification in OPSCC should assess stromal infiltration.

At present, there is an evolving dichotomy in the landscape of oropharyngeal squamous cell carcinoma (OPSCC) with human papillomavirus (HPV) emerging as an important risk factor for the development of the disease. Conversely, the influence of traditional risk factors (smoking and alcohol) and incidence of HPV-negative OPSCC is decreasing (Burgies and Cinciripini, 2007). Human papillomavirus-positive and -negative OPSCC have different molecular and clinical features (Pakhlov et al, 2008; Cancer Genome Atlas, 2015), with HPV-positive disease having a clearly established better prognosis irrespective of treatment type. This difference is likely to involve multiple factors including differential radio/chemosensitivity and genetic heterogeneity (Ang et al, 2010; Simple et al, 2013). Human papillomavirus-positive OPSCC are found more frequently at the tonsil and tongue base.
Stromal infiltration of CD8+ T cells

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sub-sites in the oropharynx, but it is unclear what factors underlie this relationship. It is possible that activation of local oropharyngeal immunity has a role in limiting the spread of the disease and/or enhancing response to therapy.

A predominant infiltration of CD3+ T lymphocytes is associated with a favourable prognosis in several cancer types (Goedert et al, 2011). However, the type and functional status of the immune cells (e.g., CD4+ cytokine effector vs tumour promoting CD4+ FoxP3+ T regulatory [Treg] cells) and the micro-environment localisation of different tumour-infiltrating lymphocytes (TILs) can determine the balance between control or promotion of cancer (Friedman et al, 2012). Recent OPSCC TIL studies have produced inconsistent findings. Studies of 46 (Wansom et al, 2012) and 83 (Nasman et al, 2012) patients reported T-cell infiltration was associated with a good prognosis and the degree of T-cell infiltration did not differ by HPV status. By contrast, other studies reported that higher CD8+ but not CD4+ T-cell infiltration was associated with a good prognosis and the degree of T-cell infiltration was positively related to HPV status (Nordfors et al, 2013; Ward et al, 2013). Ward et al (2013) investigated 270 OPSCC and found significant differences in T-cell infiltration between HPV-positive and -negative tumours with higher levels associated with a favourable outcome in HPV-positive patients.

Oropharyngeal squamous cell carcinomas have both tumour and stromal elements, which may interact to influence the biology of the cancer. Activation of the tumour stroma may drive tumour progression metabolically and/or influence inflammatory responses by modulating the balance of negative and positive immune-controlling processes (Mesureur et al, 2014). Interestingly, activation of the OPSCC stroma determined by smooth muscle actin (SMA) expression was associated with a poor prognosis, although the relationship with HPV status was not determined (Marsh et al, 2011). The stromal extracellular matrix can influence anti-tumour immunity by controlling the positioning and migration of T cells as seen in human lung tumours (Salmon and Donnadieu, 2012). A recent study of OPSCC reported that high CD8+ T-cell infiltration in the stroma was associated with a good prognosis, although, surprisingly, HPV status did not predict a better clinical outcome in this group of patients (Balermpas et al, 2013).

There is currently interest in de-escalating the treatment of good-prognosis OPSCC. Stratification based on HPV status alone may be too simplistic and underpins the need to identify additional biomarkers of outcome. Measurement of TILs is considered a promising avenue of research but requires increased understanding of the subtleties of tumour immunologic response (Iones, 2014). Therefore, the aim of the study reported here was to investigate the role of tumour microenvironment site of T-cell infiltration. The aim was addressed by investigating OPSCC from patients who were diagnosed between 2002 and 2011, and received radiotherapy. Tumour HPV status was determined using three methods and multiplex immunohistochemistry (IHC) was used to detect and enumerate different T-cell populations in tumour and stromal regions.

MATeRIALS AND METHODS

Patients. A retrospective audit using a radiotherapy database at The Christie NHS Foundation Trust Hospital identified patients with a confirmed histological diagnosis of OPSCC. Patients were treated between January 2002 and December 2011 with radiotherapy as one or the only therapy modality. Patients treated with a palliative intent were excluded. Patient clinical-pathologic and outcome data were collected from the case notes and The Christie Head and Neck assessment forms. The study was approved by the National Health Service (NHS) National Research Ethics Service committee North West (reference number 03/13/G1076). Individual patient consent was not required. Pretreatment formalin-fixed paraffin-embedded blocks prepared at biopsy were requested.

P16 expression. Tumour sections were stained with haematoxylin and eosin to confirm tumour presence before assessing HPV status. Detection of p16/cyclin D1 was used the CINtec histology kit (Roche, Basel, Switzerland) and a Biogenex ISH000 autostainer (Biogenex, Fremont, CA, USA). Human papillomavirus-positive and -negative controls were included in each staining batch. Tumours were scored as positive if there was a strong and diffuse brown staining of the nucleus and cytoplasm in ≥70% of the tumour specimen (Singhi and Westra, 2010). The slides were scored twice by a single scorer with 91% concordance. Any discrepant slides were evaluated by a pathologist to provide a final score.

In-situ hybridisation. The in-situ hybridisation (ISH) assay (Ventana Medical Systems, Tucson, AZ, USA) was performed at Manchester Royal Infirmary according to the manufacturer’s guidelines using the BenchMark automated slide-staining system. The Inform HPV III probe sets were able to detect oncoenic HPV 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68 and 70. Human papillomavirus DNA ISH slides were scored by two independent observers (80% concordance) and any discrepancies resolved by re-evaluation. A positive score was awarded only for punctate, blue-coloured staining within the nucleus of tumour cells. Diffuse staining of the nuclei was scored as a negative result.

Human papillomavirus DNA PCR. Human papillomavirus DNA-positive samples (by SPF10 DEIA) were genotyped using the INNO-LiPA HPV genotyping assay and the Roche Linear Array HPV genotyping test performed at the Institute of Cancer and Genetics, School of Medicine at Cardiff University, according to protocol.

Multiplex TIL IHC. Formalin-fixed paraffin-embedded sections 4 μm in thickness were deparaffinised in xylene and rehydrated through graded concentrations of alcohol. Following epitope retrieval (HER2) in a pressure cooker, slides were placed in a Biogenex ISH000 autostainer and endogenous peroxidase blocked using 3% peroxide solution (AcrOs Organics, Geel, Belgium).

The first multiplex staining step was DAB visualisation (brown) of FoxP3 Tα antigen. Protein block was achieved using a 10% casein solution (Vector Laboratories, Burlingame, CA, USA) before staining with the primary antibody (FoxP3, clone 286A/E7 mouse monoclonal antibody (mAb); Abcam, Cambridge, UK; 1:40). Secondary anti-mouse EnVision HRP detection system (Dako, Cambridge, UK) was subsequently used for DAB visualisation. In between slides, slides were transferred to high pH antigen retrieval solution and microwaved at 98 °C for 10 min. For the second and third stains, following peroxidase and casein blocking, CD3 (mouse mAb, clone P2.33 (Dako), 1:60) and CD4 (mouse mAb, clone 4B12 (Dako), 1:50) were visualised with the HRP chromogens ImmPRESS VIP SK-4605 (purple; Vector Laboratories) and Vector SG (blue-grey; Vector Laboratories). The fourth step was Vector Red SK-5100 visualisation (red; Vector Laboratories) of CD8 antigen. Following blocking with normal horse serum solution, CD8 antibody (mouse mAb clone C8/144B (Dako), 1:600) was applied and secondary anti-mouse Ig alkaline phosphatase (ImmPRESS, Vector Laboratories) was subsequently used to visualise Vector red. Mouse IgG1 (Dako) was used as a negative control. Finally, following multiplex IHC, sections were counterstained with haematoxylin, dehydrated in graded concentrations of alcohol and coverslipped in permanent, non-aqueous mounting.
anti-human SMA clone 1A4 (Dako), 1:500 or negative control reagent, visualization reagent and substrate-chemogen reaction solution (DAAB). Staining was scored using computer-automated H-scores (see below) and by percentage positivity from 30 randomly selected stromal regions in the single slides. Mean scores for each tumour were calculated.

**Multiplex HC automated image analysis and scoring.** For each slide, the Vectra automated multispectral imaging system (Perkin-Elmer, Waltham, MA, USA) was used to perform both low (×4) and high (×20) power scans of 30 randomly selected tissue grids. Spectral libraries were generated from single stained slides using the Nuance FX multispectral imaging system software (Perkin-Elmer). Spectral libraries and the scanned multiplex images were then loaded into inForm Advanced image analysis software (PerkinElmer). The image analysis method illustrated in Figure 1 comprised the following: (a) resolution of spectral properties of the multiplex stains; (b) classification of regions as tumour, stroma and/or blank spaces; (c) identification of different cell types; and (d) scoring based on the constituent spectral properties. The individual biopsy TIL density/region of interest (ROI; 3.5 mm²) was determined from 30 randomly selected ROIs of tumour or stromal areas for each section. The mean scores for the whole biopsy, tumour and stroma were generated. The median T-cell density for each group was used to stratify patients into 'high' or 'low' TIL groups. Automated image analysis was used to quantify the percentage of stromal cells with positive SMA expression (percentage positivity). The staining intensity was classified as low, medium or high, and H-scores were generated by multiplying percentage positivity and staining intensity scores.

**Data analysis.** Image analysis data were exported to Microsoft Excel workshrots. Charts and data comparisons were performed using SPSS version 20.0 IBM, Portsmouth, UK) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For the box plots shown, Shapiro-Wilk normality test was used to test for the distribution of the data. As the data were mostly not normally distributed, the Mann-Whitney test for comparing the data was used. Actuarial calculations of locoregional control (LRC) and overall survival (OS) were obtained using the Kaplan–Meier method. Univariate analysis was compared using the log rank (Mantel–Cox) method. The p-value was used to compare categorical data and the threshold for statistical significance was 0.05.

**RESULTS**

**Patient characteristics.** Formalin-fixed paraffin-embedded tumour biopsies were available from 218 OPSSC patients. Of these, 139 biopsies showed concordance of HPV-positive or negative phenotyping by all three detection methods and had sufficient material for further analysis. Concordance rates for HPV detection or non-detection by the three methods was 78%. Supplementary Figure S1 is the consort diagram for the study. The 5-year LRC and OS rates for the cohort were 66% and 52%, respectively. The univariate survival analysis of the patient cohort was stratified by different clinico-pathological features is summarised in Supplementary Table S1. Smoking, pretreatment haemoglobin, tumour stage, differentiation and HPV status were associated with LRC and OS. Figure 2 also shows the better LRC (P=0.004) and OS (P=0.0003) of HPV-positive compared with negative tumours.

**Infiltration of T cells.** The mean density of CD4+ T cells in the 139 biopsies (both tumour and stromal areas) was 5.2×10⁴/ROI of which 60% (3.1×10⁴) were CD4+ and 29% (1.5×10⁴) CD8+; only 0.02% (0.06×10⁴) of the CD4+ TILs were FoxP3+. Human papillomavirus-positive compared with -negative OPSSC had significantly more CD3+ (P<0.0001), CD4+ CD8+ (P<0.0001) and CD3+ CD8+ (P<0.0001) but not CD4+ FoxP3+ (P=0.1) TILs (Table 1 and Figure 3). A similar pattern of significantly increased infiltration of CD3+, CD4+ CD8+ and CD3+ CD8+ but not CD4+ FoxP3+ T cells was seen in both tumour and stromal regions of HPV-positive compared with -negative OPSSC (Table 1 and Figure 4). There

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**Figure 1.** Illustration of the method for image analysis of the multiplex immunohistochemically stained sections. The steps involved: image acquisition and processing to obtain the actual multiplex stained image (A); composite imaging of stromal (green), tumour (red) and blank (blue) spaces (B); superimposing A and B (C); enumeration of cells using haematein (D); classification of the quantified cells based on the spectral properties and multiplex staining for tumour infiltrating lymphocytes (E); and quantification of the different T-cell populations in the different compartments as CD3+ (red), CD3+ CD4+ (yellow), CD3+ (green) or negative for both markers (blue, F).
Table 1. The mean cell density of different T-cell populations per region of interest.

<table>
<thead>
<tr>
<th>CD3</th>
<th>All</th>
<th>Tumour</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>+P5 positive</td>
<td>7.9 x 10^2</td>
<td>1.5 x 10^3</td>
<td>4.2 x 10^5</td>
</tr>
<tr>
<td>P5 negative</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>1.6 x 10^5</td>
</tr>
<tr>
<td>P5 positive</td>
<td>1.8 x 10^5</td>
<td>5.0 x 10^5</td>
<td>1.3 x 10^7</td>
</tr>
<tr>
<td>P5 negative</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>+FoxP3 positive</td>
<td>5.1 x 10^2</td>
<td>2.3 x 10^3</td>
<td>2.4 x 10^5</td>
</tr>
<tr>
<td>P5 positive</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>+FoxP3 positive</td>
<td>0.1 x 10^2</td>
<td>0.006 x 10^3</td>
<td>0.005 x 10^5</td>
</tr>
<tr>
<td>P5 negative</td>
<td>0.1</td>
<td>0.2</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Abbreviations: +P5 tumour, papillary/medullary; +FoxP3 regulatory T-cell population; ROI region of interest.

TILs and clinical outcome. There was no difference in OS and LRC for the 119 patients when stratified by median CD3+ TIL density (Figure 5A). Supplementary Table S2 shows the distribution of patients according to high vs low overall CD3+ TIL density. Infiltration was significantly lower in higher American Joint Committee on Cancer prognostic staged tumours (P = 0.02) and HPV-negative tumours (P = 0.02; Supplementary Table S2). Sub-stratifying by HPV status showed that a high CD3+ T-cell density was associated with significantly better OS in HPV-positive (P = 0.02) but not in HPV-negative (P = 0.31) patients (Figure 5B and C). There was no statistically significant difference in LRC for high vs low CD3+ T-cell infiltration in either HPV-positive or -negative OPSCC. Further analysis of HPV-positive OPSCC indicated that higher CD3+ T-cell infiltration in the stroma (LRC, P = 0.03; OS, P = 0.01) but not the tumour (LRC, P = 0.75; OS, P = 0.15) regions (Figure 5D and E). Interestingly, this improved OS (Figure 5F) and LRC (Supplementary Figure S2) was related to significant stromal, and not tumour, CD3+ CD8+ but not CD3+ CD4+ TILs in HPV-positive OPSCC (Supplementary Table S3).

Stromal activation. Stromal SMA expression (Supplementary Figure S3) was significantly greater in HPV-positive compared with -negative tumours (Supplementary Figure S4) but was not associated with clinical outcome (LRC, P = 0.34; OS, P = 0.79; Supplementary Table S4). There was no relationship between the percentage positivity of SMA expression and stromal CD3+ TIL density in all patients (Supplementary Table S5). However, a significant correlation was seen in HPV-positive but not in -negative OPSCC stroma for CD3+ (P = 0.01) and CD3+ CD8+ (P = 0.0005) T-cell density Supplementary Figure S5.

Discussion

The OPSCC cohort investigated here had similar OS and LRC rates and clinico-pathological prognostic factors as described previously by others (Gillison et al, 2008; Ang et al, 2010; Oguseljor et al, 2013). To address potential inadequacies of assessing tumour HPV status in published studies, the three most common HPV detection methods (DNA PCR, DNA ISH and p16 IHC) were used and only those patients with concordant results (78%) were included for study. In the 119 patients with concordant results, 53% were classified as HPV positive, a value similar to that reported previously in OPSCC (Hama et al, 2014). It is pertinent to note that differing positivity rates have been reported globally with rates as low as 20% in the Netherlands to as high as 72% in parts of the United States (Rietbergen et al, 2013).

A strength of the study was the use of multiplex IHC and automated image analysis, which allowed for delineation of specific T-cell populations and reproducible/representative quantitative scoring. This approach enabled not only the extraction of more information from a single slide but also the observation of contextual relationships between detected antigens. The ability to localise the different immune cell phenotypes to different compartments thus adds a layer of complexity and novelty to the study of TILs in OPSCC. The study showed that patients with HPV-positive OPSCC with high CD3+ CD8+ T-cell infiltration in stromal areas have the best clinical outcome, which highlights the importance of assessing the tumour microenvironment compartment in TIL studies.

Higher TILs were found in HPV-positive compared with -negative OPSCC. This finding agrees with several (Nasman et al, 2012; Nordfors et al, 2013; Ward et al, 2014) but not all (Wassom et al, 2012; Balarupu et al, 2014) studies. Significantly higher densities of CD3+, CD8+, CD3+ CD8+ but not CD4+ FoxP3+ T-cells were detected in HPV-positive compared with negative OPSCC as a whole in this study. A further key observation was that this significantly higher infiltration was seen...
Figure 3. Scatter plots showing the differential infiltration of the T-cell subsets in HPV-positive (n = 74) and -negative (n = 65) oropharyngeal tumours. CD3+ (A), CD3+CD8+ (B) and CD3+CD4+ (C) had significantly higher infiltration of tumour-infiltrating lymphocytes in HPV-positive cancers. There was no difference in CD3+FoxP3+ cells (D).

Figure 4. Scatter plots of the differential infiltration of T-cell subsets in the various compartments (tumour and stroma) stratified according to HPV status. CD3+ (A), CD3+CD8+ (B) and CD3+CD4+ (C) T cells showed a consistent significantly higher infiltration in the tumour and stromal compartments of HPV-positive tumours. CD4+FoxP3+ (D) showed no difference in T-cell infiltration in the different compartments according to HPV status.

In both tumour and stromal regions, it is apparent that evaluation of TIL density should be done in different tumour microenvironments (Fridman et al., 2012). Stromal cells have been reported to secrete cytokines which contribute to the recruitment of TILs to the tumour micro-environment (Gajewska et al., 2013). It is presumed that the immune cell infiltration into tumours occurs in response to tumour-specific antigens and an attempt to control tumour growth and spread. However, immune-suppressive factors around the tumour periphery and/or expressed by the tumours can also attract Treg cells able to inhibit otherwise potentially active effector T cells. In this study, no difference was seen in the infiltration of Treg (CD4+FoxP3+) populations as stratified by...
Figure 5. Kaplan-Meier plots of OS of 139 OPSCC in relation to T-cell infiltration and tumour microenvironment compartment. The panels show low vs high CD3+ T-cell infiltration in all 139 (A), 74 HPV-positive (B) and 65 HPV-negative (C) tumours. D and E Survival of HPV-positive patients by CD3+ T-cell infiltration in the tumour and stromal compartments, respectively. F and G Survival of HPV-positive patients in relation to infiltration of CD3+CD8+ and CD3+CD25+ T cells, respectively, and the significantly better survival seen with higher CD3+CD8+ T-cell infiltration (P = 0.05). H and I Survival of patients with HPV-positive tumours with infiltration of CD3+CD8+ T cells in tumour and stromal compartments, respectively. Stromal infiltration but not tumour infiltration was associated with significantly better survival (P = 0.02).

HPV status, although the numbers of these cells detected were very small. Indeed, CD4+ FoxP3+ T cells were often absent from many of the scanned images (<4 per section) increasing uncertainty about the value of quantitative comparisons. There is evidence by others that high levels of systemic Treg cells are a positive prognostic marker in OPSCC (Khvorostina et al, 2014). Lukenova et al, 2014). The approach used in this paper of evaluating Treg cells in the local tumour environment did not show any prognostic relationship. It is possible that FoxP3+ may not mark all Treg cells and/or that other immune cells such as macrophages or myeloid derived suppressor cells, which were not assessed, could act to limit CD8+ T-cell activity (Damuzu et al, 2014; Caroni et al, 2015).

As expected and reported widely, HPV-positive compared with -negative OPSCC patients had a significantly better OS (P = 0.0003) and LBC (P = 0.0037). In contrast with other reports where T-cell density correlated with survival in both HPV-positive and -negative patients (Nasman et al, 2012; Wansom et al, 2012; Balempus et al, 2014), no difference was found in outcomes when stratifying all patients by total CD3+ T-cell infiltration. The heterogeneity in the patients with oral cancers studied, robustness of HPV detection and the use of a variety of IHC methods may have contributed to discordance between various published reports. However, T-cell infiltration was significantly associated with survival in HPV-positive but not in -negative OPSCC patients as reported in another study (Ward et al, 2013). In addition, our observations suggest that the stromal infiltration of CD3+CD8+ T cells is important in determining prognosis. This is consistent with a hypothesis that the higher infiltration of CD8+ T cells in the stroma is a marker of an effective immune response in HPV-positive OPSCC, contributing to improved outcome following standard therapy. In cervical dysplasia, lesion regression has been associated with T-cell infiltration of the epithelium (Trimble et al, 2010), but there is evidence that the stroma is the first point of call for the effector immune cells (Kobayashi et al, 2004; Trimble et al, 2010; Maldonado et al, 2014). Gene analysis of micro-dissected specimens of pre- and post-vaccination dysplastic lesions showed an increase in genes associated with effector immune cell phenotype, polarisation, function and activation in the stroma of post vaccination patients (Maldonado et al, 2014).

In addition to TILs, the activation status of the stroma was investigated using SMA expression by activated myofibroblasts. When present at sites of chronic inflammation, myofibroblasts promote angiogenesis, extracellular matrix, growth factor and cytokine expression (Suzuki et al, 2007). Smooth muscle action expression has been associated with a poor prognosis in several cancer types. In patients with SCC of the tongue, Kellermann et al (2007) observed higher SMA expression at the invasive margin.
significantly correlated with invasion into blood vessels, lymph node and neurons. Stromal features predicted disease mortality in oral cancer, although the proportion of OPSCC and HPV positivity was not stated (Marti et al. 2011). Our results showed a significantly higher expression of SMA in the stroma of HPV-positive compared with -negative OPSCC, but there was no association with survival. The differing numbers, heterogeneity of pathology, therapy and follow-up of patients with oral cancers studied, robustness of IVD detection and variations in IHC methodology may all have contributed to the discordance between the various published reports.

There are further levels that can influence immune control in cancer patients through co-stimulatory inhibitory pathways. The programmed cell death protein 1 (PD-1) is expressed on activated T cells and its ligands PD-L1 and PD-L2 on antigen-presenting cells (APCs) and sometimes tumour cells. The infiltration of PD-1-expressing lymphocytes can be a marker of favourable prognosis in HPV-positive OPSCC (Badoual et al. 2013) but localisation of PD-L1 to the tumour stroma interface or other immune cell types (e.g., macrophages) might limit functional tumour infiltration (Loy et al. 2013). Trials investigating the blockade of PD-1 receptors in a range of cancers (melanoma, colorectal cancer, non-small-cell lung cancer and renal cancer) have shown clinical activity (Gubin et al. 2014; Herbst et al. 2014; Powles et al. 2014; Tumeh et al. 2014). In this study, the relationship between tumour micro-environment, expression of PD1, PD-L1 and PD-L2 by TILs, tumour cells and/or APC was not evaluated. It is hypothesised that the presence of TIL in the tumour compartment may subject the suppressive effects of the co-stimulatory factors. Future studies using a multiple marker approach could investigate this relationship.

In summary, there is a growing body of evidence for an underlying immune activity in OPSCC, which is more important for HPV-positive compared with -negative disease. The work reported here highlights the importance of evaluating its context-specific nature in relation to the immune cell localisation and functional orientation. As stated in the Introduction section, the move towards patient stratification based on HPV status alone may be too simplistic, with measurements of TILs considered a promising avenue for biomarker development (Jonas et al. 2016). Our work supports this suggestion but, in addition, shows the need to assess microenvironmental localisation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

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Supplementary Information accompany this paper on British Journal of Cancer website (http://www.nature.com/bjc)
Chapter 10
Viral antigens as targets for prophylactic and therapeutic intervention in cancer
Peter L Stern and Kenneth Oguejiofor

Introduction: viral antigens and cancer immunity
Prophylactic vaccines to prevent pathogenic viral infections have had an immense impact on public health in the past century (Garcón et al., 2011). There is now a firmly established viral aetiology for around 12% of human cancers, including infections with human papillomavirus (HPV), hepatitis B (HBV), hepatitis C (HCV), Epstein-Barr virus (EBV), human T-lymphotropic virus (HTLV) and Kaposi's sarcoma-associated herpes virus (KSHV). These viruses cause many different types of cancer with the attributable fraction of that type varying from a small fraction of T-cell leukaemias with HTLV to 100% of cervical cancers (Table 10.1) (Cohen et al., 2011; Forman et al., 2012; Schiller and Lowy, 2010; Wu et al., 2012). Immunosuppression is associated with increased incidence of many of these virally associated cancers, including through the influence of human immunodeficiency virus (HIV) as well as implicating polyomaviruses in certain rare cancers (Dalianis, 2012). An understanding of the natural history of the virus infection leading to the development of cancer and in particular the role of the viral genes can provide opportunity for immunological intervention to prevent or treat disease.

The successful development and implementation of HBV prophylactic vaccination is the precedent for formulating vaccines to other oncoviruses. HBV causes a wide range of liver disease from self-limiting to chronic hepatitis. The disease pathology derives from cellular immunity to the viral products of infected cells with chronic infection leading to cirrhosis and eventually hepatocellular carcinoma (HCC). An HBV vaccine based on yeast production of a recombinant S protein, which self-assembles into lipid membrane-containing particles mimicking the S particles found in HBV infected patients, has provided excellent long-term protection against all eight viral genotypes through induction of neutralizing antibodies. HBV vaccination has had a substantial impact on the rates of acute hepatitis and on viral carrier rates in countries with well-established vaccination programmes. The prevention of chronic infections in infancy, the cause of much adult HBV-HCC, has led to dramatic reductions in the risk for this liver cancer. This article will focus mainly on the progress and challenges in the development of preventative or therapeutic immune
strategies against HPV-associated cancers. The development of immune strategies against other oncoviruses is more limited and are discussed elsewhere (Chia et al., 2012; Gourtzoulis et al., 2012; Martorelli et al., 2012; Schiller and Lowy, 2010; Smith et al., 2012; Sokal et al., 2007; Wu et al., 2012).

### HPV and cancer

HPV infection is the definite human carcinogen for six types of cancer: cervix, penis, vulva, vagina, anus, and oropharynx (including the base of the tongue and tonsils). Global estimates of the incidence of these cancers for 2008 are 610,000, with a population attributable fraction (PAF) for HPV infection of 4.8% although this varies greatly geographically (e.g. 6.9% versus 2.1% in less versus more developed regions). Cervical cancer accounts for 530,000 (86.9%) of the HPV-attributable cases with the other five cancer types accounting for the residual 80,000 cancers. Cervical cancer is the third most common female malignancy and shows a strong association with the level of country development (Forman et al., 2012). Twelve HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) are defined by the World Health Organization (WHO) as being high-risk cancer-causing types with additional types (68, 73) being recognized as 'possibly' cancer causing. Recent studies also suggest that variant viruses may differ in risk of persistence and association with high-grade premalignant disease. It appears that deregulation of viral gene expression may occur to different extents at the different sites of high-risk HPV infection, and that squamo-columnar junctions, such as the cervical transformation zone, are particularly prone to neoplastic disease. Types 16 and 18 of HPV are the most oncogenic and prevalent, being associated with more than 70% of cervical cancers. Nevertheless, high-risk HPV's do

<table>
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<tr>
<th>Virus</th>
<th>Cancer type</th>
<th>Cases/year (x1000)</th>
<th>Attributable fraction (%)</th>
<th>Mechanism/oncogene</th>
</tr>
</thead>
<tbody>
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<td>54</td>
<td>Chronic inflammation/ X-protein</td>
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<td>100</td>
<td>Oncogenic virus/multiple oncogenes</td>
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not cause cancer in the vast majority of the individuals that they infect. HPV differs from other common viral pathogens by having an intrinsically stealthy life cycle that occurs entirely within the target epithelium with no systemic infection or viremia. This poses unique challenges for vaccines designed to protect against or treat persistent HPV infections. Pre-clinical studies have established that antibodies to animal papillomaviruses can be protective against infection, and HPV oncoprotein products can be used as targets for inducing tumor immunity in animal models. An understanding of the viral life cycle, the natural immune control of infection, and escape in HPV-associated neoplasia has provided the platform for the development of prophylactic and therapeutic HPV vaccines (Stern and Kitchener, 2008).

Natural history of virus infection and oncogenesis

The HPV viral life cycle has recently been reviewed in detail (Dourdir et al., 2012). The HPV genome (8 kb) encodes eight proteins: early proteins E6, E6, and E7 are involved in cell proliferation and survival (E6 and E7 play a key role in HPV-associated carcinogenesis); three other early proteins (E1, E2, and E4) are involved in control of viral gene transcription and viral DNA replication; two late proteins L1 and L2 are involved in assembly of the virus particles. The virus particle of 55 nm diameter is made of 72 capsomers each composed of major L1 and minor L2 capsid proteins. The virus has evolved a replication cycle that is intimately linked to the life history of the differentiating epithelial cell where the small number of HPV genes coordinate in function with the host cell’s own mechanisms to support viral replication and the production of large numbers of infectious virions.

Virus infection of basal epithelial cells involves several steps (Day et al., 2010). Experimental models suggest that infection requires access of virus particles to the basal lamina, and the interaction with heparin sulphate proteoglycans. Structural changes in the virion capsid, which includes furin cleavage of L2, facilitate transfer to a secondary receptor on the basal keratinocyte, which is necessary for virus internalization and subsequent transfer of the viral genome to the nucleus where it exists as a non-integrated circular episome of less than 100 copies per cell. As these infected basal cells undergo cell division, the viral genome replicates and becomes equally segregated between the two daughter cells enabling maintenance of the HPV genome in this cell layer. New virus production is inhibited in the initial basal target cells and the productive infection process only begins with their migration upwards as suprabasal cells generate the tissue architecture and maintain its constant renewal. In uninfected epithelium the upward migration of basal cells triggers their exit from the cell cycle and they enter the pathway of terminal differentiation.

HPV relies upon the host cell for provision of key replication enzymes and other factors necessary to replicate its own genome, and it is the HPV E6 and E7 proteins that stimulate continued proliferation of the infected cells and continued availability of the replication machinery allowing the time and conditions for the virus to replicate its own genome. Once the virus has amplified its genome, to levels of many thousands of copies per cell, the HPV life cycle then switches to production of the capsid proteins L1 and L2. This stage is controlled by the early E2 protein that downregulates E6 and E7 protein expression
by blocking the binding of transcription factors to the early virus promoter allowing the process of terminal differentiation. New virions are thus assembled in the uppermost cells of the lesion that undergo apoptosis and are sloughed off as the tissue is renewed from below. If all the progeny of an initially infected basal cell differentiate with no reinfection then the HPV infection will be self-limiting, while if the basal target is a stem cell then the viral episomes can provide for a latent infection, manifest only with appropriate epithelial differentiation.

Most HPV infections clear within months, but when persistent there is an increased chance of integration of HPV genome into the host DNA and this most frequently functionally deletes the E2 gene that controls the expression of E6 and E7 oncoproteins. The increased oncogene expression acts to prevent terminal epithelial differentiation and to stop virion production. The HPV oncogenes E6 and E7 also modulate p53 and Rb mechanisms, respectively, that provide signals for exiting the cell cycle allowing opportunity for repair of host DNA. The consequence is an increase in genomic instability with accumulation of genetic mutations that can cause cellular immortalization and sometimes malignant transformation. Persistent oncogenic-type HPV infection is a necessary initial event, but not a sufficient cause of cervical cancer, that is a relatively rare outcome of a multistep process occurring over many years. Immune control of persistent HPV infection is vital to reduce the development of anogenital cancers. This is a battle between immune attack/ control and viral defence/escape, the key features of which are summarized in Boxes 10.1

**Box 10.1: Immune control of HPV infection**

- Detection of damage through innate immune system
- Activation of immediate non-specific effectors; secretion of interferons
- Pro-inflammatory cytokines and chemokines support optimal activation of local antigen-presenting Langerhans' cells, viral target antigen processing and migration to locoregional lymph nodes
- Optimal activation of adaptive immunity and generation of specific CD4 T helper 1 type immunity supporting development of effector and memory CD8 cytotoxic T cells against viral E2, E6 and E7. Cell-mediated immunity is believed to be critical in clearance of virus in basal epithelial cells
- T helper cells also support optimal activation of B cells secreting HPV capsid type-specific neutralizing antibodies. Long-lived plasma cells providing high levels of antibodies can protect against a subsequent infection through transudation into the mucosal secretions or through serous exudation
- Multiple HLA-restricted cytotoxic T lymphocytes specific for viral early antigens traffic to the lesion and target virus-infected cells to provide immune control and viral clearance
Box 10.2: Loss of immune control and escape in cervical neoplasia

- E6- and E7-mediated protection against interferons, downregulation of TLR9 and HLA expression
- Immature APC presentation of viral antigens inducing immune tolerance
- Reduction of number and activation of Langerhans' cells in intraepithelial lesions inhibiting activation of adaptive immune response
- Immune activation is skewed toward T helper 2 responses with cytokine balance supporting differentiation and infiltration of T regulatory cells that limit therapeutic T-cell effector mechanisms. The balance of local immune infiltration in persistent infection and chronic stimulation of the immune system with viral antigens can anergize controlling effector responses. HPV-specific T cells may also fail to gain access to the dysplastic epithelium
- Integration of the virus with the host cellular genome blocks the productive life cell cycle, encourages immortalization, and generates opportunity for acquisition of mutants that may offer additional means to escape immune control
- Frequent mutational events in neoplasia include HLA loss of expression making the tumor cells invisible to the CTL restricted by particular allelic genes but still resistant to NK cells

and 10.2 and extensively discussed and referenced in recent reviews (Stern and Einstein, 2012; Stern et al., 2012).

Natural immune control of an HPV infection results from the coordinated actions of the innate and the adaptive mechanisms delivered by specific antibodies and cellular effectors (e.g., T cells). Appropriate innate immune activation is critical to recruitment of the adaptive immune response that brings specific T cells to the lesions to clear the infection and provide for immune memory. T helper responses also support B cells to make virus-neutralizing antibodies that can potentially protect against future infections, although T-cell effectors are probably essential for eliminating virus-infected cells. While most infected individuals clear their HPV infections naturally, others are at risk of persistent infection. The lack of any significant cytopathic effect in the process of virus infection and production limits innate immune activation, which together with low levels of viral gene expression in the lower layers of the epithelium, can prevent or significantly delay the adaptive immune response to the initial HPV infection, and thus favor viral persistence. Constitutive expression of the E6 and E7 oncoproteins can provide for chronic antigenic stimulation of the specific T cells in anogenital neoplasia. There can be a lack of the necessary co-stimulation by local antigen-presenting cells (APCs) in the HPV lesions and this can induce an anergic response. Further, T regulatory cells (Treg), which usually
function as a part of the homeostatic control of immune responses, can also be attracted/induced by the local microenvironment and contribute to tolerance of viral antigens and the promotion of neoplasia. The oncogenic HPV genes also directly modulate the functional potential of the innate immunity through Toll-like receptor (TLR) 9 downregulation and adaptive immune effectors through reduced human leukocyte antigen (HLA) class I expression and inhibition of the interferon response pathways. The latter strategies can provide for lesion escape even in the presence of HPV oncogene-specific T cells with curative potential. In dysplastic lesions that are likely to regress, CD8+ T cells can access the epithelial compartment. However, in persistent disease, although there can be a dense immune cell infiltration, there is a failure to access the lesional epithelium. This appears to be the consequence of downregulated expression of vascular endothelial adhesion molecules that is a mechanism for human tumors to evade locally recruited immune effector cells (Stanley et al., 2012).

**Prophylactic vaccine strategies**

Protection induced by prophylactic vaccines is largely, if not entirely, mediated by antibodies that prevent virus infection. Box 10.3 summarizes the features required for a successful preventative HPV vaccine. Prevention of HPV 16 and 18 infections can be achieved by using recombinant L1 capsid proteins which form non-infectious virus-like particles (VLPs) that mimic the natural virions and provide suitable immunogens for induction of neutralizing antibodies. Since natural infection does not induce sufficient antibody levels

**Box 10.3: Requirements for a vaccine to meet the challenges of preventing HPV infection and cervical cancer**

- Virus-like particles closely mimic the virus structure – induction of neutralizing antibodies, the major basis of vaccine-induced protection
- Greatest possible protection against cervical cancer-causing HPV types – include HPV-16 and -18 VLPs as these types cause > 70% cervical cancer
- An immune response that improves on natural immunity – as natural serological immunity does not guarantee protection
- As boostability of B-cell memory by natural infection is unknown, maximal longevity of antibody levels is needed to maintain protective immune responses throughout sexually active life
- Imprinted lifespan model (Amano and Slifka, 2010) suggests that plasma cells are imprinted with a predetermined lifespan based on the magnitude of B-cell signalling that occurs during induction of an antigen-specific humoral immune response – magnitude and longevity are likely to be significantly influenced by adjuvants
for protection in most individuals, antibody levels greater than natural levels will be required. Importantly these need to present locally, which can occur by passive transudation from the blood to cervicovaginal secretions, and they need to last for a lifetime of sexual activity. The use of adjuvants is the key to maximizing the levels and longevity of neutralizing antibody induction (Garcion et al., 2011; Stern and Kitchener, 2008).

There are two licensed prophylactic vaccines. Gardasil® (Merck & Co., Whitehouse Station, NJ USA) is a quadrivalent vaccine containing VLPs of HPV types 6, 11, 16 and 18 (made in recombinant yeast) adjuvanted with amorphous aluminium hydroxypophosphate sulphate. Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) is a bivalent vaccine containing VLPs of HPV types 16 and 18 (recombinant baculovirus/insect cell production) with an adjuvant system, AS04, composed of aluminium hydroxide and monophosphoryl lipid A (MBL). MBL is a ligand for TLR4, one of the key innate immune triggers, and its mechanism of action is illustrated in Figure 10.1 (Didierlaurent et al., 2009).

Both vaccines have been extensively evaluated, including in pivotal phase III clinical trials in young women (Brown et al., 2009; Lehtinen et al., 2012; Munoz et al., 2010; Wheeler et al., 2012). Both vaccines exhibited excellent safety and immunogenicity profiles (Schiller and Lowy, 2010; Schiller et al., 2012; Stanley et al., 2012; Stern and Einstein, 2012). The vaccines also demonstrated virtually 100% efficacy against the vaccine-targeted types for a range of cervical endpoints, from persistent infection to cervical intraepithelial neoplasia grade 3 (CIN3) in women naïve to the corresponding type at the time of

**Fig. 10.1. Mode of action of AS04.**

Source: data from Didierlaurent AM et al., AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity, The Journal of Immunology, Volume 183, Number 10, pp. 6186-6197. Copyright © 2009 by The American Association of Immunologists, Inc. All rights reserved. DOI: 10.4049/jimmunol.0901474.
vaccination. CIN2/3 are histologically defined lesions that require excision treatment if detected by screening programmes. There are some different properties, with the bivalent vaccine providing increased cross-protection against oncogenic non-vaccine types, while the quadrivalent vaccine can offer protection against types HPV 6 and 11 associated with genital warts. However, the vaccines are not therapeutic, showing no effect on prevalent infection or disease. Immunogenicity-bridging studies showed excellent safety and strong immune responses in adolescents, which together with evidence of longevity in antibody responses and protection in young adults, underwrote the likely effectiveness in adolescent vaccination programs (Schiller et al., 2012). The principal target of the latter has been the prevention of cervical cancer, which provides the largest burden of preventable HPV-associated malignant disease.

However, the dividends of such HPV vaccination require sufficient coverage (~80%) and this has been challenging in many counties, including the US. School-based programmes in Australia and the UK have been very successful in delivering the necessary high coverage vaccination of adolescent girls. Early benefits will be a reduction in high-grade pre-cancers identified in screening programmes with a reduction in costs associated with treatment, but a major reduction in cervical cancer rates in women aged 20–29 years will not be apparent until 2025 and beyond. Such vaccination will almost certainly protect against other HPV-associated lesions. Clearly this could also benefit males but for national immunization programmes, as yet, vaccination of boys is not deemed cost effective compared to increasing the coverage of adolescent girls for yielding the reduction of health burden of HPV in the population. More challenging is the ability to provide vaccination to the developing world where most cervical cancer cases occur, and this will depend on the provision of more affordable vaccines as well as the means to target those at risk.

The HPV VLP vaccines are clearly highly efficacious, but as yet no minimum level of antibody required for protection has been determined and the role of B-cell memory, if antibody levels drop, is not established. The virtually 100% seroconversion of vaccinees, the high levels of antibodies induced, and the remarkable short-term efficacy has complicated attempts to define an immune correlate of protection. The design of the clinical studies in terms of the cohort size and relatively short follow up times (approximately 4 years) of the pivotal clinical trials limits the power to determine protection against lower prevalence HPV-type associated pre-cancers and to detect breakthrough infections over longer times. Only long-term follow up of vaccinated cohorts in human populations can provide data to answer such questions unequivocally. In spite of these continuing requirements for monitoring and the need for changes in other clinical practice, which will ensue from vaccination (e.g. screening), it is apparent that the current vaccines will have a profound influence on anogenital cancer. Since 2006, the two vaccines have each been licensed in over 100 countries and at the start of 2012, introduced into national immunization programs in at least 40 countries. Different health care systems and infrastructures have resulted in varied implementation strategies, with some countries delivering vaccines in schools and others through health centres or primary care providers. The GAVI (Global Alliance for Vaccines
and Immunisation) decision in 2011 to support HPV vaccination should increase implementation in low-income countries (Kane, 2012).

Against this background the prospects for the development of second-generation vaccines is obviously challenging. One approach in clinical development is to include up to nine VLP types in the vaccine formulation to increase the protection against a greater proportion of cervical cancer. However, data on the bivalent vaccine show levels of protection against 93% of high-grade CIN regardless of HPV type. This is consistent with the ability of antibodies induced against HPV 16 and 18 VLPs, adjuvanted with ASO4, to cross-neutralize related HPV types 31, 33, and 45. These five types account for up to 84% of squamous-cell carcinoma and 98% of adenocarcinoma of the cervix. Another approach involves the use of the minor capsid L2 protein that can generate neutralizing antibodies that are protective against a broad range of mucosal and cutaneous HPVs, suggesting that an L2 vaccine could act as a pan-HPV vaccine (Karannam et al., 2009). However, L2 is poorly immunogenic compared to the L1 VLPs and strategies to enhance L2 immunogenicity using multimerization, epitope display, and adjuvants are in progress (Iagu et al., 2009).

**Therapeutic vaccines strategies**

Unfortunately, most women worldwide will not receive prophylactic vaccination, particularly those who already have persistent HPV infections who are at greatest risk for developing cancer. More than 20 years ago immunologists focusing on cancer immunotherapy considered HPV-associated neoplasia as a prime target for therapeutic vaccine development against the HPV oncoproteins E6 and E7. This optimism derived from unequivocal evidence for the requirement of these oncoproteins to drive cervical cancer. While there have been large numbers of diverse vaccine approaches to generating effector T cells validated in pre-clinical models (Klebanoff et al., 2011), there has been limited translation into patients with at best low immunogenicity in either anogenital premalignant lesions such as CIN or definitive cancers (Ma et al., 2012; Stern et al., 2012; Trimble and Frazer, 2009). This partly reflects the inadequacy of the animal models for testing such vaccines since they do not reflect the complexity of the tumor and immune evolution that occurs in the natural history of an HPV-associated anogenital lesion.

The role of the microenvironment of the lesion is now known to be of central importance, but it is very difficult to measure any vaccine effects locally and the kinetics of induced responses are not necessarily predictable in a way that is consistent with clinical trial protocols. With hindsight, measuring immune responses in the peripheral blood would not necessarily predict resolution of any anogenital lesions as the importance of local infiltration of the T-cells types appears critical to the resolution or progression of disease (Stanley et al., 2012). A clear challenge is to alter the balance of the local immunity in favour of viral clearance or tumor elimination. This is likely to require induction of sufficient strength and breadth of HPV-specific CD4+ and CD8+ T-cell responses plus modulation of the lesion microenvironment to allow immune clearance.
It is now clear that the magnitude and diversity of immune infiltration can be a critical prognostic in cancer (Bindea et al., 2011). An early study in cervical cancer found a significant association between the density of tumor-infiltrating lymphocytes (TILs) and the risk of nodal, local, and distant involvement (Bethwaite et al., 1996). Subsequent studies (Gadducci et al., 2012) suggest that predominant CD4+ and/or CD8+ TILs are associated with favourable outcome, while predominant infiltration by T<sub>reg</sub> is linked with adverse outcome. This is exemplified by two recent studies that showed a successful treatment of about 50% of patients with HPV-16-induced high-grade lesions of the vulva where there was a direct correlation with vaccine-induced immune response. One vaccine was an adjuvanted set of synthetic long overlapping peptides of HPV-16 E6 and E7 (HPV-16 SLP) (Melief, 2012), and the other a recombinant HPV-16 E6E7L2 fusion protein (TA-CIN) given after local imiquimod treatment (Duayana et al., 2010). Imiquimod acts through a TLR7 that can stimulate not only local innate immunity with potent anti-tumoral effects but also drive an adaptive immune response in secondary lymphoid tissues by activating tissue APCs. Efficacy was associated with the induction of a strong HPV-specific CD4<sup>+</sup> T-cell response as well as HPV-16-specific CD8<sup>+</sup> T-cell activity. Importantly, the patients with unresponsive lesions showed reduced systemic vaccine responses as well as increased numbers of lesion-associated immune suppressive T<sub>reg</sub>. The HPV-16 SLP was also quite immunogenic in patients with cervical cancer but it failed to induce tumor regression (Melief, 2012). The failure to cure some premalignant lesions and cancers appears to result from an unfavourable balance in effector T cells and T<sub>reg</sub>. The future success of immunotherapies will need to address the means to alter this balance (Figure 10.2). Chemotherapy

**Fig. 10.2.** HPV versus immune response.
or radiotherapy can influence immune regulatory activity and in combination with vaccination may potentiate effective local HPV-specific T-cell immunity (Stern et al., 2012).

HPV-associated oropharyngeal squamous cell cancers (OPSCC), particularly cancers of the base of tongue and tonsil, have recently shown an ‘epidemic’ rise in non-smoking, non-drinking young patients, in spite of the overall decline in head and neck cancer incidence in most parts of the world. It is believed this reflects a sexual transmission of high-risk type HPV infection leading to oropharyngeal cancer. HPV-16 E6/E7 mRNA expression and more often integration with the cellular genome are found in such cancers. They are distinct from HPV-negative head and neck squamous cell cancers with regard to risk-factor profiles, molecular genetic alterations, population-level incidence trends over time, and prognosis. Thus, HPV status is the strongest determinant of survival for patients with local-regionally advanced oropharyngeal cancer, but whether this relates to the proximity of a network of lymphoid tissues is unknown (Gillison et al., 2012). It has been suggested that improved outcomes are associated with increased TILs and this is independent of HPV status (Wansom et al., 2012). On the other hand, others have reported that although increased TILs were associated with better prognosis independent of HPV status, there were higher numbers of infiltrating CD8+ T cells in HPV tumors (Nasman et al., 2012). These observations highlight the complexity of the factors influencing the control of HPV-positive OPSCC. Interestingly, it was recently shown that chemoradiotherapy of head and neck cancers resulted in a reduced tumor Treg density and an increased ratio of T effector cells locally; importantly this correlated with better survival outcomes (Tabachnyk et al., 2012). These data suggest an opportunity for improving the immunogenicity of cancer vaccination. This potential for combination of conventional and immunotherapeutic treatments is supported by pre-clinical data showing low-dose radiation combined with an HPV E7 DNA vaccine incorporating calreticulin resulted in improved tumor therapy (Tseng et al., 2009).

Provision of effective immune therapies for HPV metastatic cancers is still a major challenge. Our increased understanding of the role of immune regulation in limiting effective anti-tumor responses, particularly lesion infiltration of effectors, the plethora of strategies to improve the immunogenicity of HPV vaccination, and the optimal combination use with chemoradiation may provide progress in the future. Given the inadequacy of animals models for HPV-associated cancer, only clinical trials will be able to test therapies integrating such knowledge, and the challenge is to assess 'the where and the when' for the combination of treatments, the effects on the immune system, and the tumor microenvironment (Stanley et al., 2012; Stern et al., 2012).

References


