Genetic analyses, protein expression, type and function with clinical and immunological correlates in human papillomavirus associated head and neck neoplasia

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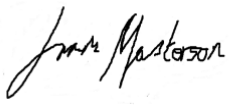
Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text

It does not exceed the prescribed word limit for the Doctor of Medicine Degree Committee.

Signed: 

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Summary

In head and neck squamous cell carcinoma (HNSCC), the evidence that human papillomavirus (HPV) is associated with a subgroup of tumours has increased over the last thirty years. Prospective randomised controlled clinical trials have now established that detection of HPV in oropharyngeal tumours (~60-70% of cases in North America and Europe) may confer a survival advantage to the patient.

Within the oropharynx, HPV16 constitutes ~90-95% of HPV subtypes associated with malignancy. This contrasts with uterine cervix mucosa, where approximately 15 high-risk subtypes cause >99% of disease. Other factors such as differences in genetic background, host immune response, hormonal and environmental influences (e.g. tobacco smoke, alcohol) all play a part in the pathway and susceptibility to oncogenesis.

Analogous to uterine cervix disease, HPV-associated cancers involve wild-type TP53 whilst HPV negative tumours often have mutations in this gene.

As most patients with HPV associated OPSCC present at an advanced stage, the detection and genetic analysis of a pre-malignant state would be important as it infers the potential for a screening test (similar to the uterine cervix model). To investigate this, whole transcriptome analysis with verification of results by reverse transcription–quantitative polymerase chain reaction (PCR), was performed on OPSCC fresh tissue biopsy samples. Predictable fold changes of RNA expression in HPV-associated disease included multiple transcripts within the p53 oncogenic pathway (e.g. *CDKN2A* / *CCND1*). In addition to this, a testis-specific gene not normally expressed in somatic cells, SYCP2, showed a consistently elevated fold change from baseline in pre-malignant and malignant tissue.

A subtle immune defect has long been thought to trigger the susceptibility of some individuals to persistent HPV infection with either low or high risk HPV types. Following on from this, we investigated if clinical outcomes in HPV-related head and

neck cancer may be affected by host immune response. Peripheral blood from patients with OPSCC treated by chemoradiotherapy underwent IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT) to examine cell-mediated immune responses to HPV16 E2, E6 and E7. T cell responses against E6 or E7 peptides correlated with HPV DNA/RNA status. Within the HPV16+ OPSCC cohort, enhanced immunoreactivity to antigen E7 was linked to improved survival. In addition, an observed increase in regulatory T cell frequencies after treatment would suggest that immunosuppression may contribute to a reduced HPV specific cell mediated response.

A further aspect of this study was to determine the role of HPV and Epstein Barr Virus (EBV) in the pathogenesis of squamous cell carcinoma within the temporal bone region. This is an uncommon tumour which is normally preceded by a history of inflammation within the external auditory canal (EAC) or middle ear / mastoid cavity. Although HPV has been implicated in many head and neck malignancies, its role in SCC of the temporal bone has not been established. Treatment strategies could change if a viral aetiology can be found. HPV16 DNA was detected in ~20% of the cases studied, however, no significant difference in disease specific survival was noted for the papillomavirus positive group. Epstein-Barr virus was not detected.

The data presented highlight the functional and biological influence of high risk HPV infection on HNSCC. Further studies are likely to focus on developing non-invasive screening tools, therapeutic strategies based on vaccination or immune modulation or indeed de-escalation of current treatment protocols.

Abbreviations

ATP	adenosine triphosphate
bp	base pair(s)
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid disodium salt dehydrate
EGF	epidermal growth factor
ELISPOT	enzyme-linked immunospot
FCS	foetal calf serum
FFPE	formalin fixed paraffin embedded
FISH	fluorescence *in situ* hybridization
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic DNA
H&E	haematoxylin & eosin
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HMBS	hydroxymethylbilane synthase
HPV	human papillomavirus
HR	homologous recombination
HSIL	high-grade squamous intraepithelial lesion
HSV-2	herpes simplex virus type 2
hTERT	human telomerase reverse transcriptase
IARC	International Agency for Research on Cancer
IL	interleukin

IFN-γ	interferon- γ
kbp	kilobase pairs
LR-HPV	low-risk human papillomavirus
LSIL	low-grade squamous intraepithelial lesion
MHC	major histocompatibility complex
mRNA	messenger RNA
nm	nanometer
nt	nucleotides
OPSCC	oropharyngeal squamous cell carcinoma
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
RTqPCR	real time quantitative polymerase chain reaction
RIN	RNA integrity number
RNA	ribonucleic acid
RT	reverse transcriptase
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TBE	tris borate EDTA
UICC	Union for International Cancer Control
URR	upstream regulatory region
UV	ultraviolet
VLP	virus like particle
WTS	whole transcriptome sequencing

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As a clinician, entering the mysterious world of laboratory medicine was a truly daunting step. Without doubt, this transition was made possible by the patience and good nature of the fantastic people I have met.

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Finally, the future of oncology will almost certainly benefit from the rapid advances made in genomic medicine and immunotherapy. This project has allowed me a unique insight into both these fields and I hope to continue this further with the 100K genome head & neck collaboration.

Publications

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Presentations (* = Invited presentation ** = National *** = International)

1. CD8+ T-cell response to HPV16 E7 predicts survival outcome in oropharyngeal cancer. *BAHNO Annual Conference* May 2016 (London, UK)**

2. De-escalation treatment protocols for HPV associated oropharyngeal carcinoma. *30th International Papillomavirus Conference* Sept 2015 (Lisbon, Portugal) * / ***

3. Investigating pre-invasive and invasive epithelial changes in human papillomavirus associated oropharyngeal carcinoma – whole transcriptome analysis results from the UKCRN11945 trial. *5th IFHNOS conference* July 2014 (New York, USA)***

4. Cell mediated immune response in HPV associated oropharyngeal squamous cell carcinoma. *5th IFHNOS conference* July 2014 (New York, USA)***

5. Expression profile differences in human papillomavirus associated and non-associated oropharyngeal carcinoma *European Congress on Head & Neck Oncology* April 2014 (Liverpool, UK)***

6. Pre-malignant biomarkers in HPV associated oropharyngeal carcinoma *Royal Society of Medicine* March 2014 (London, UK)**

7. Oropharyngeal carcinoma, HPV & screening *Clinical Fellows Meeting CRUK Annual Conference* March 2013 (London, UK)**

8. The association of temporal bone squamous cell carcinoma with human papillomavirus. *14th British Academic Conference in Otolaryngology* July 2012 (Glasgow, UK)***

Prizes

British Association of Endocrine and Thyroid Surgeons (BAETS) Travelling Fellowship "*Thyroid & Parathyroid Surgery*" 2016 (Hamburg 2016)

International Federation Head Neck Oncology *Young Investigator Award* (New York 2014)

Tse Cheuk Ng Tai *Prize in Skull Base Research* (Cambridge 2014)

European Congress on Head Neck Oncology *Young Investigator Award* (Liverpool 2014)

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Introduction

1.1 Squamous cell carcinoma of the oropharynx

1.1.1 Anatomy

The oropharynx is one the most important functional sites within the head and neck region as it is located at the bifurcation of the respiratory and digestive tract. Tumours at this site will affect speech, swallowing and ultimately the airway. Decisions about treatment are influenced not only by the optimal method of tumour ablation, but also by important functional considerations.

The oropharynx is divided into four anatomical subsites and extends from the level of the soft palate above to the dorsal surface of the epiglottis below (**Figure 1, Table 1**). Tumours may arise in the oropharynx from different epithelial elements and three main histological types are recognised:

1. Squamous cell carcinoma – tumour arising from squamous epithelium
2. Lymphoma – arising from lymphoid follicles in the tonsils or base of the tongue
3. Salivary gland tumours – derived from minor salivary glands sited mainly at the soft palate or capsule of the tonsil

As noted in other sites within the head and neck region, the most common cancer encountered in the oropharynx is squamous cell carcinoma which accounts for >90% of cases (Mehanna, 2011). Non-Hodgkin lymphomas tend to occur more frequently in the oropharynx compared to other sites in the head and neck because of the higher concentration of lymphoid tissue. Salivary gland tumours and other rare tumours account for <5% of all cases.

Figure 1 Anatomical zones of the pharynx (*Picture courtesy of Mayo Clinic Foundation, USA*).

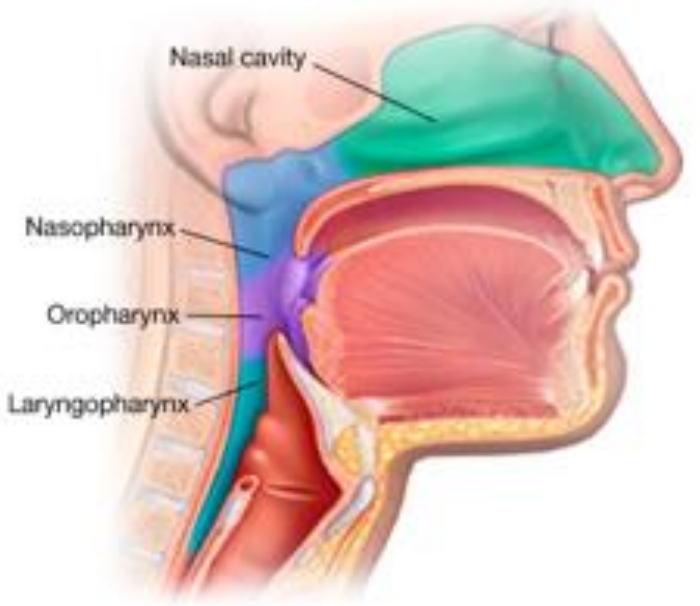


Table 1 Oropharyngeal subsites

Subsite	Anatomical regions included
Anterior wall (oropharynx)	• Vallecula (dorsal surface of the epiglottis) • Tongue posterior to the circumvallate papillae (tongue base)
Lateral wall (oropharynx)	• Tonsil • Tonsillar fossa and faucial pillars
Posterior wall (oropharynx)	• Pharynx
Superior wall (oropharynx)	• Inferior surface of the soft palate • Uvula

1.1.2 Epidemiology

An estimated 600,000 cases of Head & Neck Squamous Cell Carcinoma (HNSCC) occur each year, making it the sixth most common cancer worldwide. In developed countries, since the 1980s, the incidence of oropharyngeal carcinoma (one of the largest subgroups of HNSCC) has dramatically increased, and there has been a change in the disease demographic towards younger aged males with less tobacco consumption (de Martel *et al*., 2012).

Risk factors traditionally associated with development of Oropharyngeal Squamous Cell Carcinoma (OPSCC) include tobacco and excess alcohol consumption, which have a synergistic effect and are responsible for most global disease.

The worldwide incident rate of OPSCC is 3.8 per 100,000 population. In Europe, some countries have a higher rate than the global average e.g. Hungary (16.9 per 100,000). The highest number of oropharyngeal cancer cases emanate from South East Asia (especially India which has an incident rate of 12.5 per 100,000) (Price *et al*., 2010, Soerjomataram *et al*., 2012).

Human papillomavirus has a causal role in almost all uterine cervix cancers, and in the last 20-30 years this same virus has become increasingly important in SCC affecting the tonsil or tongue base regions. Within the head and neck region, the role of HPV in non-oropharyngeal cancer is less well defined. A review by Mehanna *et al*. (2012) found HPV DNA was detected in 20% of SCCs affecting the larynx, oral cavity, nasopharynx and hypopharynx. However, the clinical significance of these findings is reduced when it is noted that the same detection rate has remained static in comparison to OPSCC.

There are >20 HPV types identified to have an aetiological role in invasive cancer of the uterine cervix. HPV16 and HPV18 are responsible for ~70% of uterine cervix cancer cases (Winer *et al.*, 2006), while the most predominant type associated with OPSCC is HPV16 (~95%) (Ang *et al.*, 2010). Infection with a non-HPV16 type is less likely to lead to OPSCC in contrast to cancers of the uterine cervix (Isayeva *et al.*, 2012). This would suggest that the natural history of HPV life cycle is different

according to the site (e.g. oropharyngeal crypt mucosa) and cell (e.g. keratinocyte) of infection. Other factors such as susceptibility of the site to hormonal control mechanisms, access to the site by other carcinogens found in tobacco smoke, alcohol and betel leaf chewing might also play some role.

A recent study from the National Institute of Health (USA) found an increase in the prevalence of OPSCC that are HPV-associated from 16.3% in the 1980s to 72.2% during the 2000s (Chaturvedi *et al*., 2011). A systematic review published after this study suggests that the proportion of HPV-associated OPSCC in trials recruiting before the year 2000 (40.5%) was almost half the rate for trials recruiting after 2005 (72.2%) (Mehanna *et al.*, 2012). Schache *et al*. (2011) performed the only large retrospective UK study which suggested a similar rise in prevalence rates (14% in 1998 increasing to 57% in 2009) (Schache *et al*., 2011).

In the United States, it is predicted that if published trends continue for HPV-associated OPSCC, this will overtake the annual incidence of cervical cancers by the year 2020 (**Figure 2**). This data may be influenced by the substantial decline in cervical cancer rates due to screening (Chaturvedi *et al*., 2011). The observed rise in HPV-associated OPSCC has now been confirmed in other developed countries with similar cervical cancer screening protocols (Garnaes *et al*., 2015).

Human papillomavirus is now a significant cause of oropharyngeal cancer (especially in developed countries); however, the exact prevalence will vary according to the level of tobacco-associated disease. In Europe, the proportion of HPV-positive tumours varies from ~90% in Sweden to less than 20% in communities with the highest rates of tobacco use (**Figure 3)** (Nasman *et al.*, 2015).

Figure 2A: The annual number of HPV-associated oropharyngeal cancers (in the USA) is projected to exceed that of uterine cervix cancers by the year 2020. **Figure 2B:** This dramatic change appears to be driven by a rapid rise in male cases (Chaturvedi *et al*., 2011).

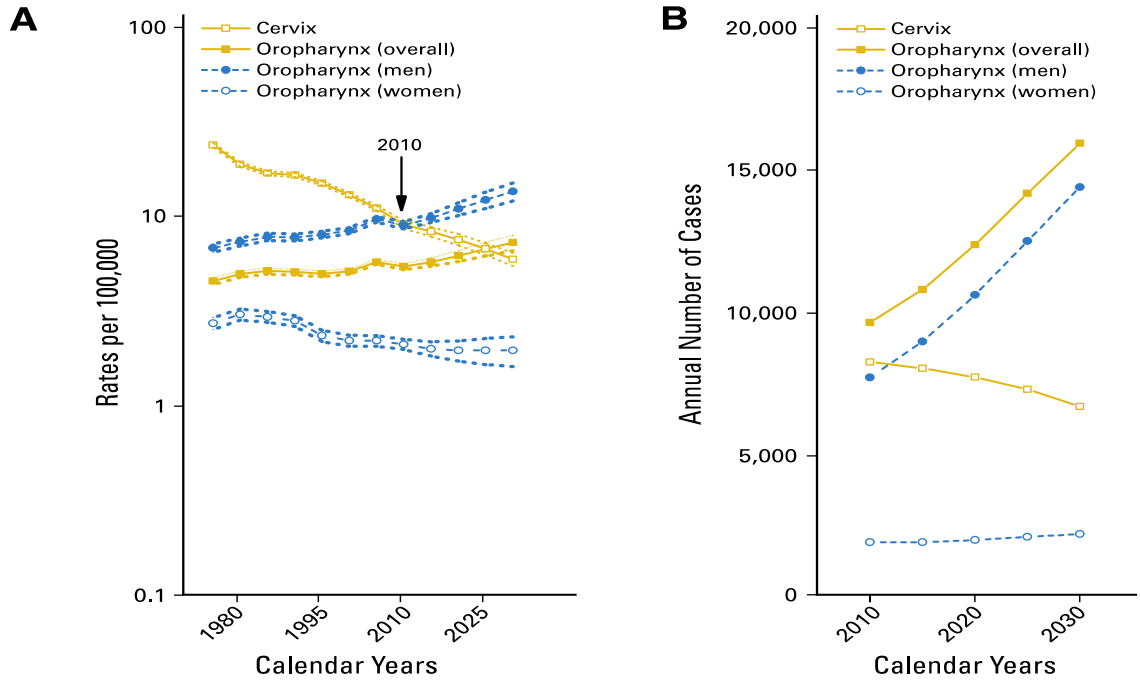
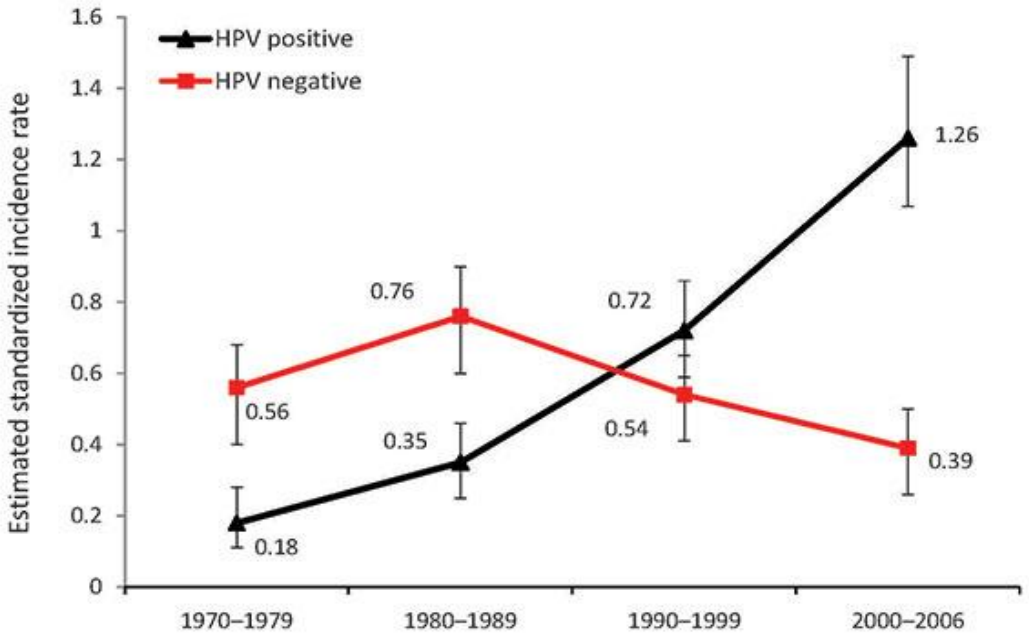


Figure 3 Swedish national cancer registry data showing an exponential rise in the incidence rate for HPV-associated tonsil cancer for the period 1970-2006 (Näsman *et al.*, 2009).



1.1.3 Established risk factors

Tobacco

Smokers have a six-fold increase in cancers compared to non-smokers. Stopping smoking may reduce the risk of OPSCC but this will take >10-20 years to reduce to the level of a non-smoker (Parkin, 2011).

Tobacco is still the prime aetiologic agent responsible for SCC of the upper aerodigestive tract. The average cigarette contains more than 4000 chemicals, many of which have been identified as toxic or carcinogenic. Burning of tobacco releases benzopyrine, methylchloranthrene and other aromatic hydrocarbons which reach the surface of the epithelium dissolved in saliva. Further breakdown of these carcinogens produces carcinogenic epoxides, which bind to DNA molecules, damaging them and initiating the process of cancer formation (Patel *et al.*, 2009).

A higher frequency of p53 mutations is observed in tobacco users, which is a key determinant of oncogenesis (Gandini *et al.*, 2008). The carcinogenic process depends on multiple factors: type of tobacco, the duration and intensity of carcinogen exposure, genetic factors, type of delivery method, epithelial mucous production, ciliary paralysis and dietary factors (Gandini *et al.*, 2008).

Alcohol

Alcohol and tobacco have a synergistic effect and can increase the risk of developing HNSCC by 20-120 times that of a non-alcoholic/non-smoker (Pelucchi *et al.*, 2008). Alcohol dehydrates the cell wall and promotes the ability of tobacco carcinogens to permeate local tissues, in addition it depletes antioxidants, which have a protective role in cancers (Seitz and Becker, 2007). The solubility of carcinogens in tobacco is increased when saliva is mixed with alcohol, and this in turn promotes absorption into the surface epithelium (Seitz and Becker, 2007, Pelucchi *et al.*, 2008). The quantity, quality and duration of alcohol consumed will determine the absolute risk in OPSCC.

HPV

There is strong evidence for the causal association of HPV infection in OPSCC (Gillison *et al.*, 2000). HPV infection is strongly associated with young, sexually active, non-smoker males and may be accountable for the changing demographic profile of OPSCC towards a younger, non-smoking population (Chaturvedi *et al.*, 2011).

Risk factors for HPV infection include oral sex practices, a large number of sexual partners and first intercourse at a younger age. Changing sexual practices associated with modern society may increase the cumulative effect of HPV infection in OPSCC (Chung and Gillison, 2009).

HPV-associated OPSCC behaves differently from the other, more common HPV-negative variety which affects an older age group and is normally associated with smoking and alcohol. These former patients often present with a neck node, respond well to radiation and have a better prognosis than the HPV-negative cohort (Fakhry *et al.*, 2008). As a result, many academic centres are investigating the potential to de-escalate existing treatments that are often associated with severe side effects (Trotti and Gillison, 2011, Masterson *et al.*, 2014). It is now known that smokers with HPV-associated OPSCC have a higher risk profile compared to non-smokers with HPV-associated malignant disease (Gillison *et al.*, 2011).

Genetic

Tobacco-associated susceptibility to oropharyngeal carcinoma can also be determined by genetic factors. It is estimated that the risk of developing OPSCC, in patients with a first-degree relative, can increase 2- to 14-fold above baseline. Patients with Fanconi anaemia (a rare inherited condition linked to leukaemia or aplastic anaemia) are also at significantly increased risk for OPSCC in comparison to the general population (Kumar *et al.*, 2013). At this stage, it is unclear if a similar genetic susceptibility to persistent HPV infection and subsequent transformation may exist.

Diet

A non-balanced diet may be associated with ~10-15% of oropharyngeal cancers. Foods derived from high calorie sources have been reported to present a higher risk e.g. starch rich pulses, processed / salted meats and eggs (Sapkota *et al.*, 2008).

Reactive oxygen species can initiate carcinogenic pathways while antioxidants enable some protection against the molecular damage. Vegetables and fruits contain naturally occurring antioxidants; other antioxidants include those derived from vitamins A and C. Dietary deficiencies, particularly of iron, selenium, folate, vitamin E, and other trace elements have been linked to increased risk of cancer of the upper aerodigestive tract (Gonzalez, 2006).

1.1.4 Clinical presentation

Symptoms

It is a regrettable fact that oropharyngeal carcinoma normally presents at an advanced stage, with >75% patients allocated to the stage III/IV category (Tables 2, 3). The underlying reason for this delayed presentation is attributed to the vagueness of symptoms such as persistent sore throat, otalgia or discomfort on swallowing. The majority of patients with HPV-associated OPSCC present with a new onset neck mass [57-79%] (Gillison *et al.*, 2011). This finding suggests that, in comparison with HPV-negative disease, these tumours metastasize to local lymph nodes when the primary tumour is relatively small

A distorted voice often described as akin to having a “hot potato” in the mouth has also been described. Tumours that are large at presentation (>T3) are often associated with gradual loss of swallowing and speech due to reduced tongue movement.

Physical examination

A thorough examination of the neck and upper aerodigestive tract is essential to determine potential malignant disease. Flexible endoscopy under local anaesthetic has greatly facilitated the ease of examination of the pharynx and larynx. Smaller tumours of the tongue base or tonsil arising in lymphatic crypts may not be detectable unless palpated under general anaesthetic. Ulcerative tumour types invade deeply, whereas, exophytic tumours spread superficially. A hypoglossal nerve paresis may cause wasting (+/- fasciculation) of the tongue with deviation to the ipsilateral side on protrusion.

Systematic palpation of the neck must be performed to detect nodal spread from OPSCC. Malignant disease is typically fixed, irregular and firm but can masquerade as inflammatory lymph node enlargement if examined at an early stage (Mendenhall *et al*., 2006). Neck palpation can be difficult to perform in patients with a large neck or muscular spasm with a 30% inaccuracy rate reported by many studies (Dayanand *et al.*, 2010). Tonsil SCC drains primarily to jugulodigastric, submandibular and upper posterior triangle nodes. Further spread to the deep cervical chain (levels II, III and IV) is also characteristic with occasional involvement of the carotid sheath or retropharyngeal region. Bilateral lymphatic spread (levels II-IV) occurs in approximately a third of patients with tongue base involvement (Woolgar, 2007).

Investigations

Any suspicious node in the neck should undergo Fine Needle Aspiration Cytology (FNAC) or core biopsy provided no contra-indications exist. This may allow rapid differentiation between an SCC, lymphoma or a branchial cyst.

Detection of metastatic nodal disease is more sensitive with ultrasound than palpation alone and has a specificity of ~75%. This improves to >90% if combined with FNAC / core biopsy (van den Brekel and Castelijns, 2005).

Lesions, which are confirmed or highly suspicious for malignancy, will need further investigation in the form of a neck + chest computed tomography (CT) scan +/- Magnetic Resonance Imaging (MRI). This is mandatory to assess the size of the primary tumour and accurately evaluate the neck to determine presence, size, site and invasive potential of malignant lymph nodes. MRI has an advantage for assessing small lesions due to enhanced soft tissue definition whereas CT is better for assessing bony invasion to the mandible (Weber *et al.*, 2003).

Fluoro-2-deoxy-d-glucose positron emission tomography (FDG-PET) is a useful imaging technique that can demonstrate an increased concentration of radio-nucleotide tracer in malignant tissue. This works on the basis that malignant cells are metabolically more active than the cells within normal adjacent tissue. Accumulation of the FDG tracer can also occur in areas of inflammation or infection leading to possible confounding results (Al-Ibraheem *et al.*, 2009).

FDG-PET now has an established role in the detection of a second primary or distant metastasis in locally advanced OPSCC. The integrated use of FDG-PET and CT is also deemed more accurate than either alone for detection and anatomic localisation of head and neck malignancy (Jwa *et al.*, 2012). Meta-analysis data (Xu *et al.*, 2012) indicate PET/CT to have a specificity of 96% and sensitivity of 83% for detection of distant malignant disease, whereas for conventional imaging, the values are 96% and 44% respectively.

Accurate response assessment after chemo-radiotherapy (CRT) is essential treatment in patients with advanced disease. PET/CT is optimally timed after 12 weeks of completion of treatment, but can be done sooner if there is clinical suspicion of recurrence. In a meta-analysis of 51 studies, the reported negative predictive value (NPV) for both primary and nodal disease response assessment was 95% (Gupta *et al.*, 2011).

1.1.5 Staging

The American Joint Committee on Cancer (AJCC) / *Union for International Cancer Control (UICC)* criteria are now the most accepted classification for OPSCC (Edge *et al*., 2010). Distant metastases are unusual at presentation but may eventually occur in ~10% of patients, which is second only to hypopharynx subsite. The lungs are the commonest region to be involved followed by spread to bones or the liver (Yao *et al.*, 2012). HPV-associated OPSCC can also rarely present with brain metastases (Ruzevick *et al.*, 2013). In 2017, the AJCC / UICC released the 8th Edition of the staging manual for head and neck malignant disease (Amin *et al*., 2017). This created a separate algorithm for HPV-associated cancer of the oropharynx to give a more accurate prediction of survival for newly diagnosed patients (**Table 2**; see also section 1.1.6). At the time of writing this thesis, the British Association of Head & Neck Oncologists still recommend the widespread adoption of the 7th TNM classification until a consensus meeting has been convened (Mehanna, 2018).

Table 2: Changes to TNM classification of primary oropharyngeal squamous cell carcinoma (Amin et al., 2017).

		TNM version 7 (p16+/- OPSCC)		**TNM version 8** (p16+ OPSCC only)
Tum. (T)	**T1**	Tumour 2 cm or smaller in greatest dimension	**T1**	Unchanged
	T2	Tumour larger than 2 cm but 4 cm or smaller in greatest dimension	**T2**	Unchanged
	T3	Tumour larger than 4 cm in greatest dimension	**T3**	Unchanged
	T4a	Tumour invades the larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate or mandible	**T4**	Moderately advanced tumour invading larynx, extrinsic tongue muscles, medial pterygoid, hard palate or mandible or beyond
	T4b	Tumour invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx or skull base or encases carotid artery		
Node (N)	**N1**	Metastasis in a single ipsilateral lymph node, 3 cm or smaller in greatest dimension	**N1**	Metastasis in a single ipsilateral lymph node, 6 cm or smaller in greatest dimension
	N2a	Metastasis in a single ipsilateral lymph node larger than 3 cm but 6 cm or smaller in greatest dimension	**N2**	Metastasis to contralateral or bilateral lymph nodes, 6 cm or smaller in greatest dimension
	N2 b	Metastasis in multiple ipsilateral lymph nodes, 6 cm or smaller in greatest dimension		
	N2c	Metastasis in bilateral or contralateral lymph nodes, 6 cm or smaller in greatest dimension		
	N3	Metastasis in a lymph node larger than 6 cm in greatest dimension	**N3**	Metastasis in any cervical lymph node >6 cm in greatest dimension
Met. (M)	**M0**	No distant metastasis	**M0**	No distant metastasis
	M1	Distant metastasis	**M1**	Distant metastasis

TNM version 7: **Stage I:** Describes a small tumour (T1) with no spread to lymph nodes (N0) and no distant metastasis (M0). **Stage II:** Describes a tumour that is smaller than 4 cm (T2) and has not spread to lymph nodes (N0) or to distant parts of the body (M0). **Stage III:** Describes a larger tumour (T3) with no spread to lymph nodes (N0) or metastasis (M0), as well as smaller tumours (T1, T2) that have spread to regional lymph nodes (N1) but have no sign of metastasis (M0). **Stage IVA:** Describes any invasive tumour (T4a) with either no lymph node involvement (N0) or spread to only a single, same-sided lymph node (N1) but no metastasis (M0). It is also used for any tumour (any T) with more significant nodal involvement (N2) but no metastasis (M0). **Stage IVB:** Describes any tumour (any T) with extensive nodal involvement (N3) but no metastasis (M0). **Stage IVC:** Indicates there is evidence of distant spread (any T, any N, M1).
TNM version 8: **Stage I:** T1 or T2 + N0 or N1 + M0. **Stage II:** T1 or T2 + N2 + M0 / T3 or T4 + N0 or N1 + M0. **Stage III:** T3 or T4 + N2 + M0. **Stage IV:** Any T or N + M1.

1.1.6 Treatment

The current treatment of OPSCC depends on clinician/patient preference and stage of the disease (**Table 3**). Radiotherapy or surgery are modalities usually employed for early disease (T1 N0 / T2 N0). For more advanced lesions, treatment usually involves primary chemoradiation (+/- neck dissection) or surgical excision (+/- free flap reconstruction +/- adjuvant chemoradiotherapy if adverse histological features) (Mehanna, 2011).

Table 3: Oropharyngeal squamous cell carcinoma stage at presentation. The data indicate most patients are diagnosed at an advanced stage (III/IV), which may be attributed to the vagueness of symptoms such as persistent sore throat, otalgia or discomfort on swallowing. In addition, HPV-associated disease is more likely to present with cystic nodal disease.

Study	**Stage I (%)**	**Stage II (%)**	**Stage III (%)**	**Stage IV (%)**
(Masterson and Tanweer, 2013)	4.4	7.4	19.3	68.9
(Bhide *et al.*, 2008)	0.8	1.6	37.2	60.5
(Preuss *et al.*, 2007)	8.6	14.8	22.5	49.2
Average	**4.6**	**7.9**	**26.3**	**59.5**

HPV-associated OPSCC patients are usually younger, non-smokers, with improved physiological reserve compared to HPV-negative counterparts (Posner *et al.*, 2011). In addition, Ang *et al.* (2010) found their HPV-positive OPSCC group to be associated with a Caucasian ethnicity and small (<T2) primary tumour size.

With these observations in mind, how can the response to treatment in patients with HPV-associated OPSCC be explained? One possibility is that HPV-associated disease is less aggressive than tobacco- or alcohol-induced carcinoma. Most clinical studies assessing outcome in OPSCC observe that HPV status is an independent variable predicting tumour response; this is regardless of treatment modality if it is delivered per the standard of care (Licitra *et al.*, 2006).

The improved clinical outcome observed in HPV-associated OPSCC is generally ascribed to a distinct biological pathway. Treatment of HPV-associated cells with cisplatin and fluorouracil chemotherapy will repress HPV E6 and E7 viral oncogene expression and re-instate the p53/pRb tumour suppressor pathways (Leemans *et al.*, 2011). This contrasts with tobacco- or alcohol-induced cancers that incorporate mutations conferring a more resistant phenotype.

At present, HPV-associated OPSCC is treated in a similar manner to HPV-negative disease, and one may argue that complications of treatment are more burdensome in this former group (long term side effects in a younger cohort could exert a higher socio-economic cost to society).

There are now several large trials currently ongoing which investigate the different options for de-escalation of treatment in HPV-positive OPSCC. The various approaches include the use of cetuximab (an epidermal growth factor receptor (EGFR) inhibitor) in place of conventional chemotherapy or reduction in chemoradiation dosing schedules (Trotti and Gillison, 2011). As HPV-associated disease is a distinct biological entity, future strategies are underway to reduce expression of the E6/E7 oncoprotein or develop vaccines that specifically target HPV16 (Jones, 2012).

Although there is an enhanced interest in HPV-associated OPSCC, it must not be overlooked that, on a global scale, most patients are still classified as HPV-negative. This latter cohort has inferior clinical outcomes, which have not changed over the past 20-30 years. One study, designed for adjuvant therapy in HPV-negative disease, utilises a modified adenovirus (ONYX-015) to target and destroy mutated p53 cancer cells (Yoo *et al.*, 2009). More recently, the SPECTRUM trial demonstrated a trend

towards improved clinical outcomes in HPV-negative OPSCC using panitumumab (EGFR inhibitor) combined with cisplatin radiotherapy (Vermorken *et al.*, 2013).

1.1.7 Clinical outcomes

The development of a second primary tumour is noted to be more likely in HPV-negative in comparison to HPV-positive OPSCC (Licitra *et al.*, 2006). As the follow-up in this study amounted to only ~40-50 months, it may be difficult to draw conclusions. However, a more recent trial with a longer observation period (median = 7 years), confirmed this observation by demonstrating that loco-regional failure rate in HPV-associated OPSCC was significantly lower than HPV-negative OPSCC [27% versus 71%; P<0.0001] (Posner *et al.*, 2011). Such information may be encouraging, although it is important to be aware that a diagnosis of one HPV-related cancer may increase the risk of developing a second HPV-related malignancy (Edgren and Sparen, 2007). When present, distant metastatic spread from HPV-associated OPSCC occurs mainly in the lungs, liver, bones or more rarely to the brain.

A comprehensive review by Genden (2013), which looked at retrospective studies in OPSCC, revealed a significantly improved survival (~25% relative risk) in HPV-associated OPSCC in comparison to HPV-negative disease.

In a study of 323 patients enrolled to a prospective clinical trial, Ang *et al.* (2010), reported a three-year survival of 57% vs. 82% for HPV-negative OPSCC against HPV-associated disease by analysing pre-treatment biopsy samples. A prospective study by Fakhry *et al.* (2008) observed that HPV-associated OPSCC had an improved overall survival, with 95% of patients alive after two years, compared to 62% of those with HPV-negative disease.

Although, short-term outcomes for HPV-associated patients may be explained by the molecular biology of the tumour, other factors may also influence the survival quoted above, i.e. patients with HPV-associated disease often tend to have an improved physiological reserve due to their younger age, and this, in turn, may increase the probability of undergoing full course treatment. Also, relatively less tobacco

consumption in the HPV-associated group may reduce the risk of field cancerisation (Gillison *et al.*, 2011). However, Ang *et al.* (2010) conclude that the survival benefit attributed to this demographic profile is likely to be small in comparison to biological factors.

1.1.8 HPV infection in the oral cavity

Kreimer *et al.* (2013) recently studied the natural history of oral cavity HPV infection for 1626 healthy male volunteers. In this study, the median follow-up of 12 months revealed that newly acquired oncogenic HPV infections were rare and most were cleared within this period.

The relationship between the practice of oral sex and oral HPV infection is a subject of increased interest. Further research has also been directed towards the practice of oral sex and the development of OPSCC. In 2012, an epidemiological study detailed an association between sexual behaviour and HPV infection within the oral cavity (Gillison *et al.*, 2012). They observed that the relative risk of HPV infection correlated with an increased number of sexual partners. There was also a significant difference in relative risk compared to those with no history of sexual contact.

A further cross-sectional study investigated the sexual practice of 1000 adults. This demonstrated a positive correlation with the number of oral sex partners, with a significantly greater risk for the subset with 5-10 oral sex partners (Pickard, 2012). However, Termine (2011) observed that the best predictor of oral HPV infection was a younger age of sexual debut and that orogenital sexual practice did not influence this outcome.

Gillison *et al.* (2012) commented that the correlation of oral sex with HPV-positive OPSCC is difficult to prove due to the co-linearity of sexual behaviours i.e. oral sex practice is frequently accompanied by other practices / factors that may increase the chance of HPV transmission. In addition, the perceived embarrassing nature of orogenital sexual behaviour may result in artificially low reporting by subjects. To

reduce this form of bias, studies now frequently utilize computers to allow "automated interviews", which guarantee anonymity (D'Souza *et al.*, 2009).

Herrero *et al.* (2003) performed a multi-site study and found that subjects reporting a history of oral sex were more likely to develop HPV-associated oropharyngeal SCC disease (OR = 3.2, 95% CI = 1.5 to 6.4). In addition, HPV DNA positive tumours were increased two-fold in subjects with >1 oral sex partner, and in subjects with >6 partners this increased to five-fold (Termine, 2011). Marur *et al*. (2010) observed that a significant minority of HPV-associated OPSCC patients reported no history of oral sex practice which suggests that this is not the only aetiological factor.

Although some observational studies have reported opposing results, a meta-analysis in 2009 concluded that OPSCC was associated with >4 lifetime orogenital sex partners and/or >6 lifetime sexual partners (Heck, 2009).

There are no studies which have investigated the natural history of oral cavity HPV infection preceding OPSCC; however, uterine cervix cancer studies demonstrate that the average length of infection is 5-15 years before progression to malignancy (Beachler, 2012).

1.1.9 HPV detection methods in OPSCC

The most accepted approach for HPV analysis is based on detection of DNA or RNA by signal or target amplification methods. HPV L1 capsid antigen and E6 / E7 oncoproteins can be detected by immunohistochemical techniques, but these are not frequently used due to lack of sensitivity (Robinson *et al.*, 2010).

Signal amplification incorporates the hybridisation of a target probe with viral nucleic acids. The resulting complex can be visualised *in situ* with either chromogenic or fluorescent *in situ* hybridisation (ISH) assays (Singhi and Westra, 2010).

The amplification of a specific nucleic acid sequence by "target" methods involves the use of primers, nucleic acid polymerases and nucleotides. The resulting

polymerase chain reaction (PCR) is now the most accepted target amplification method for HPV detection (Schache *et al.*, 2011).

At present, no standardised protocol exists for HPV detection in OPSCC (Robinson *et al.*, 2012). The gold standard test is recognised to be RNA quantitative PCR (E6 / E7) but this is difficult to achieve outside the confines of a research laboratory due to the requirement for fresh frozen rather than FFPE tissue. One observational trial recommended the use of $p16^{INK4A}$ immunohistochemistry as a surrogate screening marker for high risk HPV followed by DNA *in situ* hybridisation to increase assay specificity (Singhi and Westra, 2010).

A study from the Netherlands found that when HPV16 DNA ISH is combined with $p16^{INK4A}$ this will improve the overall sensitivity and specificity rate to 96% (Smeets *et al.*, 2007). A study from the UK also compared the DNA ISH method with $p16^{INK4A}$ IHC for detecting HPV in head and neck cancers. Although they found that the DNA ISH assay was less sensitive compared with qPCR detection of viral E6/E7, combined DNA ISH and $p16^{INK4A}$ gave an overall improved performance (Schache *et al.*, 2011).

1.1.10 Dysplastic changes preceding invasive SCC in HPV associated OPSCC

HPV-positive oropharyngeal squamous cell carcinomas have characteristic histologic features such as a basaloid morphology or lobular growth pattern. The tumours frequently show a high mitotic activity as demonstrated by cell cycle markers e.g. MCM7 or Ki-67 (Ghittoni *et al.*, 2010). HPV-associated OPSCC relates to a non-keratinizing histological appearance, which can result in the wrong allocation to a poorly differentiated SCC category. It is now accepted that HPV-associated malignant epithelium (within the oropharynx) emanates from the reticulated lining of tonsil crypts rather than squamous epithelium, and, therefore, a non-keratinized appearance supports the interpretation of a well-differentiated tumour process (Begum S *et al.*, 2005). As yet, few studies in the literature have described the association of HPV16-positive OPSCC and presence of pre-malignant lesions (Mooren *et al.*, 2014, Kreimer *et al.*, 2013).

OPSCC carcinogenesis may involve progressive transformation from normal epithelium to pre-malignant tissue (dysplasia / carcinoma in situ) to invasive cancer (Miller and Johnstone, 2001, Jayaprakash *et al.*, 2011, Leemans *et al.*, 2011, van Zeeburg *et al.*, 2012, Mooren *et al.*, 2014). Classification of dysplastic (pre-malignant) epithelium within the oropharynx is a subject of continued debate. The intra- and inter-observer variation are relatively high in comparison to uterine cervix cancer due to lack of a single validated morphological criterion and the biological differences found within tonsil and tongue base epithelium (Fleskens and Slootweg, 2009).

While the presence of HPV subtypes within invasive oropharyngeal SCC has been evaluated in large epidemiological studies (Mehanna *et al.*, 2012, Nasman *et al.*, 2015) there is limited data on this subject in regions of confirmed dysplasia / carcinoma in situ (Mooren *et al.*, 2014).

Prior studies in areas of confirmed dysplasia / carcinoma in situ have reported markedly variable HPV prevalence rates due to limitations of size and variable assay techniques (Miller and Johnstone, 2001, Chaudhary *et al.*, 2009). Jayaprakash *et al.* (2011) published a meta-analysis of 22 relevant articles, suggesting HPV16 to be present in ~25% of all dysplastic lesions within the oropharyngeal subsite – the same author concluded this to be a conservative estimate due to the inclusion of oral cavity SCC lesions in some of the studies (traditionally a subsite with low HPV16 prevalence).

As the majority (~75%) of patients with HPV-positive OPSCC present at an advanced stage with cystic nodal disease (Licitra *et al.*, 2006, Ang *et al.*, 2010), investigation of pre-malignant molecular pathways represents an important research priority, with the ultimate aim to produce a non-invasive screening tool (Psyrri *et al.*, 2012).

At present, there is a relative paucity of data regarding factors that influence progression of disease from normal epithelium to in situ carcinoma to invasive malignancy. In normal individuals, mucosal HPV carriage in the head and neck varies between 2% - 8% (Duray et al., 2011, Gillison et al., 2012). HPV16 prevalence in the oral cavity is ~1% in the US population (Gillison *et al.*, 2012). Age (peak

prevalence 30-34 and 60-64 years), sex (males >females), smoking, and multiple sexual partners were independently associated with oral HPV infection in multivariable models. The duration / persistence of oral HPV16 is most likely influenced by local and systemic immune status of an individual (discussed further in chapter 4). Initial studies suggest that most oral HPV infections are likely to be cleared within a year (Chung *et al*., 2014). In OPSCC, Thavaraj *et al*. (2014) indicate that virus-induced field cancerisation and/or multifocal oncogenic HPV infection is uncommon outside of the palatine or lingual tonsil region.

Despite the limitations mentioned above, histopathological evaluation based on light microscopic examination remains the best option for assessing malignant potential of pre-invasive oropharyngeal lesions. The WHO (Gale *et al.*, 2005) and Ljubljana (Gale *et al.*, 2000) classification systems are the most accepted in HNSCC and both follow parameters similar to those in frequent use for the grading of squamous lesions of the uterine cervix (**Figure 4**) (Baldwin *et al*., 2003). It is a matter of debate as to whether this is appropriate, as uterine cervix epithelium will display a more obvious difference between abnormal and normal epithelial layers and consequently the level of dysplasia.

1.1.11 Biomarker screening

Within the invasive epithelium of HPV-associated OPSCC, the major pathways targeted by HPV oncoproteins E6 / E7 may include p53, $p16^{INK4A}$, cyclin D1, hTERT and pRb [comprising RB1 / RBL1 / RBL2 and involved in cell cycle regulation (Leemans *et al*., 2011)]; EGFR and TGF-β [intracellular growth factor signaling (Bornstein *et al*., 2009, Sheu *et al*., 2009)]; tyrosine kinase receptors [evasion of apoptosis by PIK3CA amplification, HRAS mutation and PTEN loss (Chiosea SI *et al.*, 2013)]; Wnt / β-catenin [cadherin-catenin adhesion system (Rampias *et al.*, 2010)]; and hypoxia-inducible factor [neo-angiogenesis (Aderhold *et al*., 2013)]. HPV involvement with the above pathways can result in progressive mutations and ultimately genomic instability. As yet, limited data exists as to whether these or other similar pathways extend to dysplastic tissue surrounding viral associated OPSCC.

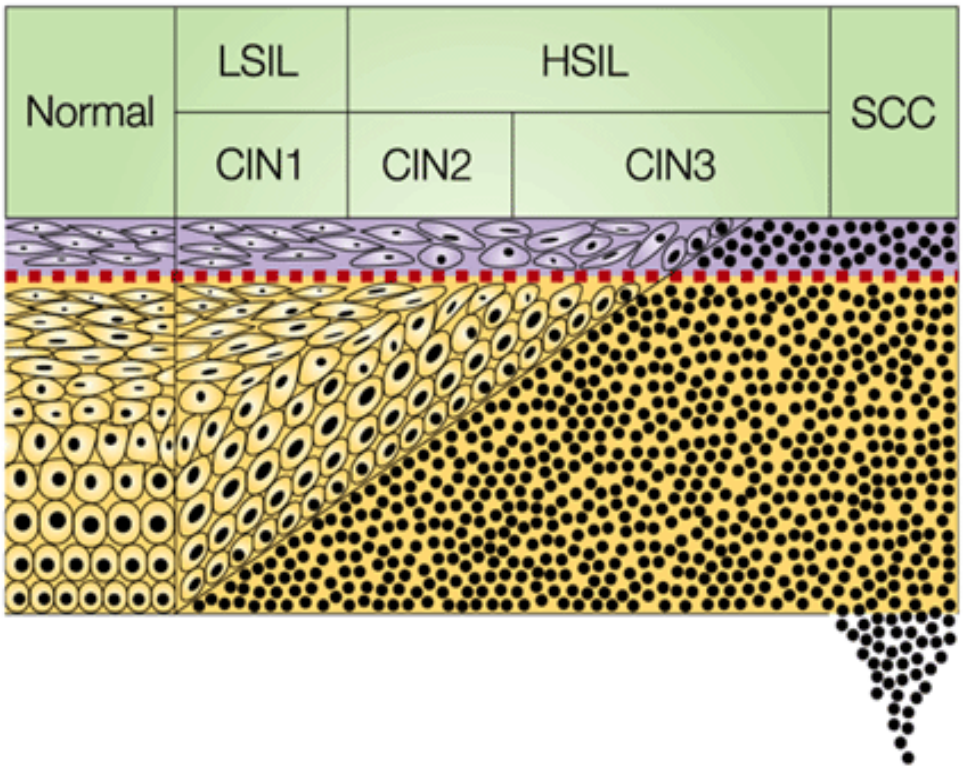
To date, data from three large genome-wide sequencing studies of HNSCCs are available (Stransky, 2011, Agrawal, 2011, Network., 2015). The results from sequencing data have been disappointing from a therapeutic target standpoint because of the relative paucity of oncogene mutations in comparison to the more-frequent tumour suppressor gene (TSG) mutations.

TSGs are poor therapeutic targets because restoring loss-of-function in these genes is more difficult than inhibiting increased activity resulting from gain-of-function in oncogenes. Of the 15 most-frequent mutations in HNSCC, only two are known oncogenes, *PIK3CA* and *HRAS*, and only *PIK3CA* is currently considered to be therapeutically targetable (Kang, 2015).

HPV-positive tumours have a lower average number of mutations per tumour, and rarely have *TP53* mutation and loss of p16INK4A function compared with HPV-negative tumours, reflecting the biological differences in these tumours (Stransky, 2011, Agrawal, 2011).

HNSCC has an immunosuppressive influence with patients demonstrating low absolute lymphocyte counts, decreased antigen-presenting function and elevated T-regulatory cells (Masterson *et al*., 2016a). HNSCC achieves immune evasion by down regulating expression of the antigen-processing molecules, TAP1/2 and MHC1. In addition, co-inhibitory receptors, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death ligand-1 (PD-L1) which induce immune tolerance to HNSCC, are frequently expressed on tumours (Ferris, 2015). The cytokine microenvironment promotes tumorigenesis with excessive immunosuppressive cytokines such as VEGF, IL-6, TGF-β, and IL-10. 18 The success of immune-checkpoint inhibitors in solid tumours, along with the increases in HPV-positive HNSCC incidence, has raised enthusiasm for novel immunotherapeutic approaches and development of corresponding biomarkers (Ferris *et al*., 2016).

Figure 4: The uterine cervical intraepithelial neoplasia (CIN) classification system is based on the proportion of dysplasia observed without invasion of the basement membrane (⅓, ⅔ and full thickness representing CIN I, II & III respectively). (Baldwin *et al.*, 2003).



1.1.12 Squamous Cell Carcinoma of the temporal bone

Squamous cell carcinoma (SCC) is the most prevalent tumour type within the temporal bone region. The reported incidence is <6 cases per million per year, which accounts for 0.3% of all cancers within the head and neck. There is a slight predisposition to the male sex, and the median age at presentation is within the seventh decade (Gidley *et al.*, 2010).

Modern skull base and reconstructive surgery techniques have improved the treatment of SCC of the temporal bone. Surgery, in the form of a lateral or extended temporal bone resection, is now more effective due to a greater understanding of skull base anatomy. Imaging techniques such as magnetic resonance (MR) and computed

tomography (CT) help produce accurate assessment of the disease extent regarding soft tissue involvement and / or bone erosion. With this approach, recent case series have demonstrated improved control of local advanced (T4) disease with five-year survival rates ~40% (Bacciu *et al*., 2013, Masterson *et al*., 2014).

Aetiology

Established risk factors for SCC within the epithelium of the temporal bone are chronic suppurative otitis media (CSOM) and previous radiotherapy (Masterson *et al*., 2014). Exposure to ultraviolet light may be a risk factor for SCC that originates in the external auditory canal (Lobo *et al*., 2008).

One theory, which may provide a connection for the various predisposing factors, is the establishment of metaplastic or neoplastic change from a chronic inflammatory disease process. Chronic otitis media and cholesteatoma are common in patients with SCC affecting the temporal bone (Gidley *et al.*, 2010). In recent years, several studies have suggested an aetiological role for high risk human papillomavirus, however, they are limited to archival samples with disparate methods of collection (Jin *et al*., 1997, Wang *et al*., 2009). As yet, no study has been conducted on a prospective basis with fresh tissue due to the infrequent nature of this condition. It is also not clear if this viral subgroup demonstrates an improved survival after treatment.

A past medical history of radiotherapy is relevant and may serve as a red flag at presentation. Goh *et al.* (1999) reported seven patients with temporal bone malignancy all of whom were initially treated by radiotherapy for other head and neck disease. The latency period before symptoms ranged from 5-30 years (Goh *et al.*, 1999). This potential risk should be considered when deciding upon the most appropriate treatment for individuals with tumours of the head and neck - especially if the patient is young and/or the condition is benign i.e. acoustic neuroma or vascular malformation.

Long term ultra-violet light exposure to the pinna or lateral concha can lead to carcinogenic change that migrates medially (Lobo *et al.*, 2008). This is more likely in

Caucasians (versus other ethnic groups) who are prone to non-melanoma skin cancers in sun exposed areas. A genetic susceptibility to skin cancer may also exist, manifesting as the development of skin cancers in sites not exposed to sunlight (in addition to more conventional locations).

Staging

The modified Pittsburg staging system predicts disease outcome by tumour size, local / distant metastatic spread and/or facial nerve involvement. A revised University of Pittsburgh staging system in 2000 suggested that temporal bone SCC with evidence of a facial nerve palsy should be categorized as T4 (Moody *et al.*, 2000). Multiple studies have since endorsed this new classification (Higgins and Antonio, 2010).

Treatment

At present, there is no consensus strategy for operative management of a temporal bone tumour. The main surgical approaches are en-bloc resection versus piecemeal resection or radical mastoidectomy (all with adjuvant radiotherapy). A proportion of oncological procedures require piecemeal tumour removal as the main segment of tissue is weakened or fragmented by the carcinoma. In patients treated outside tertiary centres, the diagnosis is often made during a mastoid exploration for a presumed cholesteatoma or other middle ear disease. In this latter scenario, a pragmatic approach (influenced by patient choice) entails postoperative radiotherapy alone, which may adversely affect patient survival.

In recent studies, the best survival outcomes are achieved with en-bloc extended temporal bone resection followed by radiotherapy (Gidley *et al*., 2010, Bacciu *et al*., 2013, Masterson *et al*., 2014). All demonstrate improved survival figures despite treating a cohort of patients dominated by T3 and T4 disease.

Outcomes

Tumours with a well-differentiated histology have a favourable outcome, with most series reporting an ~80% survival rate. Advanced stage tumours have reduced survival rates even after adequate surgery and adjuvant radiation treatment. Other adverse factors include node positive status, facial nerve palsy, positive surgical margins and carotid artery involvement (Higgins and Antonio, 2010, Masterson *et al.*, 2014).

Prior surgical intervention is often a pre-requisite towards making the diagnosis of malignancy, but this can complicate pre-operative staging, disrupt margins and require the surgeon to operate in an inflammatory region. The literature is divided as to whether persistent / residual disease will result in significantly reduced survival when compared to patients who have not received prior treatment (Moore *et al.*, 2007).

1.2 Human papillomavirus

1.2.1 The virus

Human Papillomavirus is a major carcinogen, with an estimated link to 4.8% of total worldwide cancers in 2008 (de Martel *et al*., 2012). The overwhelming majority of uterine cervix cancers (99.7%) are causally associated with HPV, but the situation is not so clearly defined for other sites within the body. The virus consists of double stranded circular DNA and is strictly epitheliotropic. There are >150 types of HPV, some of which are involved in carcinogenesis and are designated as high-risk (Leemans *et al.*, 2011).

HPV16 is associated with the significant majority of viral associated cancers within the head and neck and in particular the oropharynx subsite. In addition, strong evidence exists to suggest an oncogenic role for HPV E6 and E7 with inhibition of p53 and pRb (retinoblastoma) tumour suppressor proteins, respectively (Rautava and Syrjanen, 2012).

Expression of the E6 protein will cause inhibition of p53, leading to loss of cell cycle arrest and apoptosis when DNA damage is detected within the cell. Expression of the E7 protein will abrogate the interaction of pRb with E2F1, leading to unopposed progression through the cell cycle (Leemans *et al.*, 2011). Persistent HPV infection is pre-requisite for the establishment of this oncogenic change, and thus, the virus must evade both innate and adaptive immune defences.

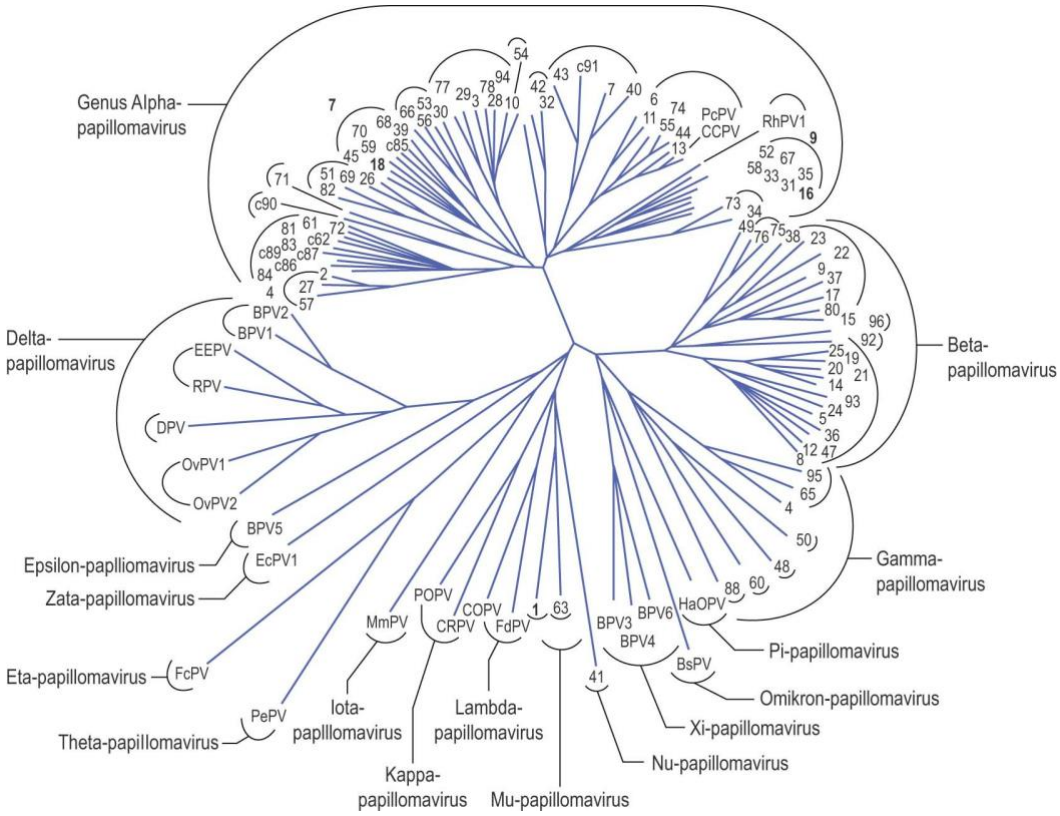
1.2.2 HPV subtypes

HPV types, subtypes and variants are categorised by variations in the L1 nucleotide sequence and will display a tropism towards either mucosal or skin surfaces. Low risk HPV types (2, 4, 6, 11, 13, 32, 42, 43, and 44) form benign papillary growths, while high risk HPV types (16, 18, 31, 33, 34, 35, 39, 45, 51, 56, 58 and 59) are correlated

with a number of pre-malignant and malignant lesions found in the anogenital and upper aerodigestive tract regions (Rautava and Syrjanen, 2012).

The papillomaviruses were designated as a distinct family of viruses in 2004. The taxonomy levels include Family e.g. papillomavirus; Genus e.g. alpha or beta; Type e.g. HPV16 (>10% difference in sequence of L1 from other HPV types such as HPV18); Subtype i.e. 2-10% dissimilarity in L1 sequence; Variant e.g. <2% dissimilarity in L1 sequence. Mucosal HPV types mainly belong to the alpha genus while the skin cancer types are primarily restricted to the beta genus (**Figure 5**) (de Villiers *et al.*, 2004).

Figure 5: The papillomavirus phylogenetic tree is classified by L1 gene sequence variation. Oncogenic (high risk) HPV subtypes are restricted to the alpha genus (de Villiers *et al*., 2004).



1.1.3 Genomic organisation and structure

The HPV viral genome is circular, non-encapsulated and has an approximate size of 8000 base pair units (**Figure 6**). The solitary DNA strand consists of 8 open reading frames (ORFs). The genome is further divided into three components, which are respectively, the early region, the late region and a non-coding upstream regulatory region (URR) (Stanley, 2012a).

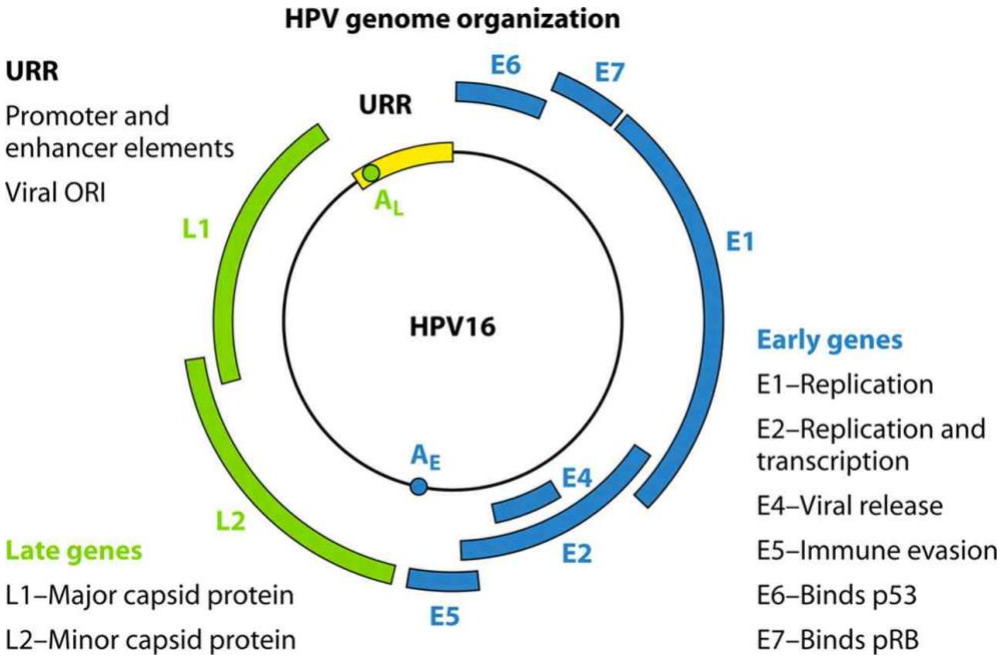
The viral proteins E1-E7 are transcribed from the early (E) region and control the transcription of viral DNA. Viral proteins L1 and L2 are transcribed from the late (L) region and control construction of the virion and egress from the host cell *(*Leemans *et al*., 2011).

The replication factors E1 and E2 function at the level of viral DNA replication and transcription. E4 is considered to be involved in the regulation of late viral functions (e.g. viral particle release), however, this exact role remains unclear (Stanley, 2012a). E5 protein affects the early stage of viral integration by influencing tyrosine kinase growth factor receptor signaling pathways. E6 and E7 genes have a role in the normal viral life cycle by targeting the host proteins pRb and p53, which in turn preserves the replicative potential of infected cells (Moody and Laimins, 2010).

Benign HPV types are associated with low risk HPV DNA in episomal form while high risk HPV DNA in malignant lesions is often, but not always, integrated within the cellular genome (Feller *et al.*, 2009). Integration of the viral genome may act to disrupt the inhibitory function of E2 leading to increased expression of E6 and E7 (Moody and Laimins, 2010). This may in turn provide a selective growth advantage to affected cells, as integrant-derived transcripts have better stability than those derived from episomal viral DNA (Rautava and Syrjanen, 2012).

However, many studies suggest that co-existence of both episomal and integrated forms can be important in HPV mediated carcinogenesis. Mixed forms of HPV16 DNA are reported in both anogenital and head & neck carcinomas (Kadaja *et al.*, 2009).

Figure 6: Genomic organisation of human papillomavirus (Stanley, 2012a).



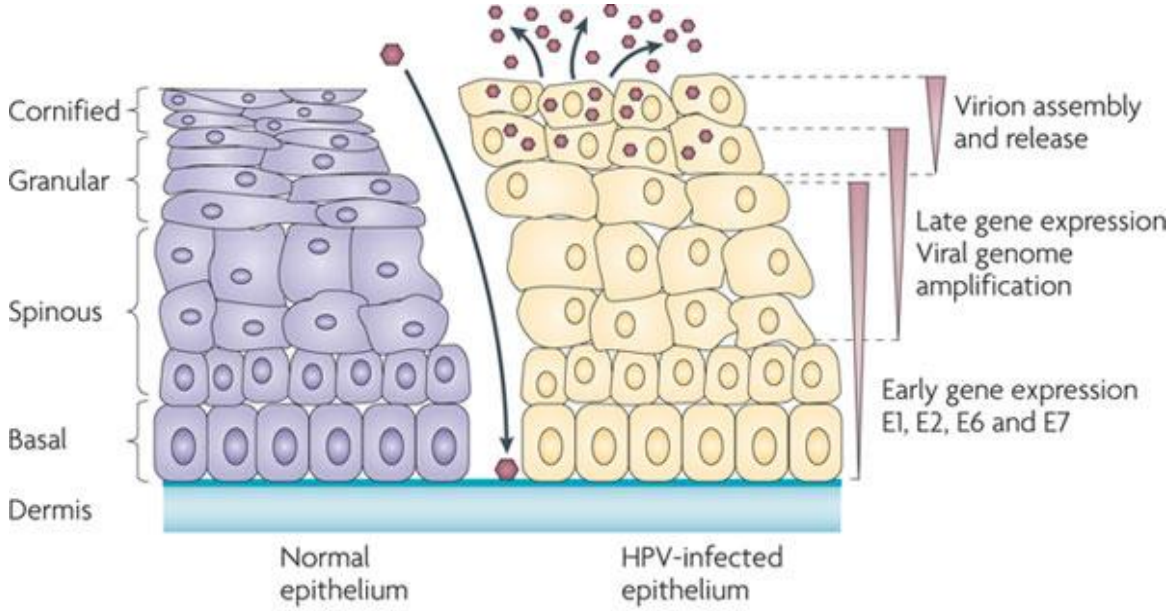
1.2.4 HPV life cycle

HPVs show selective affinity for undifferentiated keratinocytes, infecting the basal cell layer of squamous epithelium (Moody and Laimins, 2010). The virus obtains access to the basal cell region via microscopic breaks in the skin or mucosa and is maintained at this site at a low copy number (~1-10 copies per cell). Viral entry into tonsil crypt epithelium is of specific interest as this is the most prevalent HPV-associated carcinoma outside the anogenital site. The reticulated (sponge-like) lining of the crypts is important as a first line in immune defence and facilitates transport of antigens to provide a reservoir of immunoglobulins (Kim *et al.*, 2007). A propensity for defects in this epithelial lining may explain the predisposition for high risk HPV infection.

The life cycle of HPV is closely aligned with the differentiation of epithelial cells. In the productive stage of viral DNA replication, the HPV genome can be amplified significantly (~1000 copies per cell). When keratinocyte cells divide, one daughter cell begins to terminally differentiate while the other remains in the basal layer. As the differentiated keratinocyte has no ability to replicate, the virus is required to express E2 as an initiating factor. The target sequences for E2 are the E2 binding sites (E2BSs), which are in the regulatory region of most HPVs. If the viral genome has integrated, this stimulates the host cell to move from G1 to S phase, by removing the inhibitory function of E2 and increasing expression of E6 and E7 (Moody and Laimins, 2010).

The manufacture of new virus-like particles within the superficial layer of the epithelium follows late gene transcription and translation, and the affected host cells are eventually desquamated (Doorbar, 2006). This process requires expression of E1-E4, in addition to the late genes (L1 and L2), to facilitate viral DNA encapsulation and release of infectious virions (**Figure 7**).

Figure 7: HPV viral life cycle according to epithelial differentiation (Moody and Laimins, 2010).



1.2.5 Virus oncogenic activity

Amplification of the papillomavirus to levels above 1,000 copies is normally reserved for differentiated keratinocytes that are restricted from further cell division. Oncogenic (high risk) viruses have developed strategies to uncouple this cellular proliferation and differentiation primarily by expression of viral proteins E6 and E7 (Doorbar, 2006).

The exact mechanism in which high and low risk E6 and E7 proteins differ has not been fully determined, although it has been suggested that the former group has a more profound influence on cell cycle regulators (Feller *et al.*, 2009).

High risk E6 protein can inhibit the action of p53 by binding to cellular ubiquitin ligase E6AP. It also blocks the action of p21, which has a regulatory function within the cell in response to DNA damage. This dual action ameliorates the ability of the host cell to undergo apoptotic response. E6 can also lead to increased expression of hTERT (telomerase reverse transcriptase), which negates loss of telomerase and in turn facilitates cell immortalization / genomic instability.

The hypo-phosphorylated form of pRb associates with E2F1 molecules that in turn can regulate the normal cell cycle. High risk E7 can bind to hypophosphorylated pRb, abrogating its interaction with E2F1, resulting in an effect similar to that of pRb in a phosphorylated state, in which the cell is led into cell cycle (S phase). Cyclins D1-3 are activated by the resultant mitogenic signals and in turn act to promote cell cycle progression. E7 will also act to block p21 and in addition increase expression of cyclin-dependent kinase inhibitor-2A ($P16^{INK4A}$). This in turn binds to CDK4, inhibiting subsequent interaction with cyclin D and stimulating passage through the G1 phase of the cell cycle (**Figure 8**) (Ghittoni *et al.*, 2010).

As the infection continues, the E2 protein will normally induce E6 and E7 down-regulation and subsequently restore p53 induced apoptosis. Where this is not the case, both E6 and E7 will contribute to cell transformation and maintenance of a malignant phenotype. Persistent viral infection is required to achieve this, and immune evasion of HPV is primarily orchestrated by E6, E7 and E5 (**Figure 9**).

Figure 8: Schematic presentation of progression toward malignancy caused by HPV oncoproteins E6 and E7. The diagram is restricted to oncological pathways only - E6 and E7 have many other interactions with cell proteins that can affect the cell cycle (Burd, 2003).

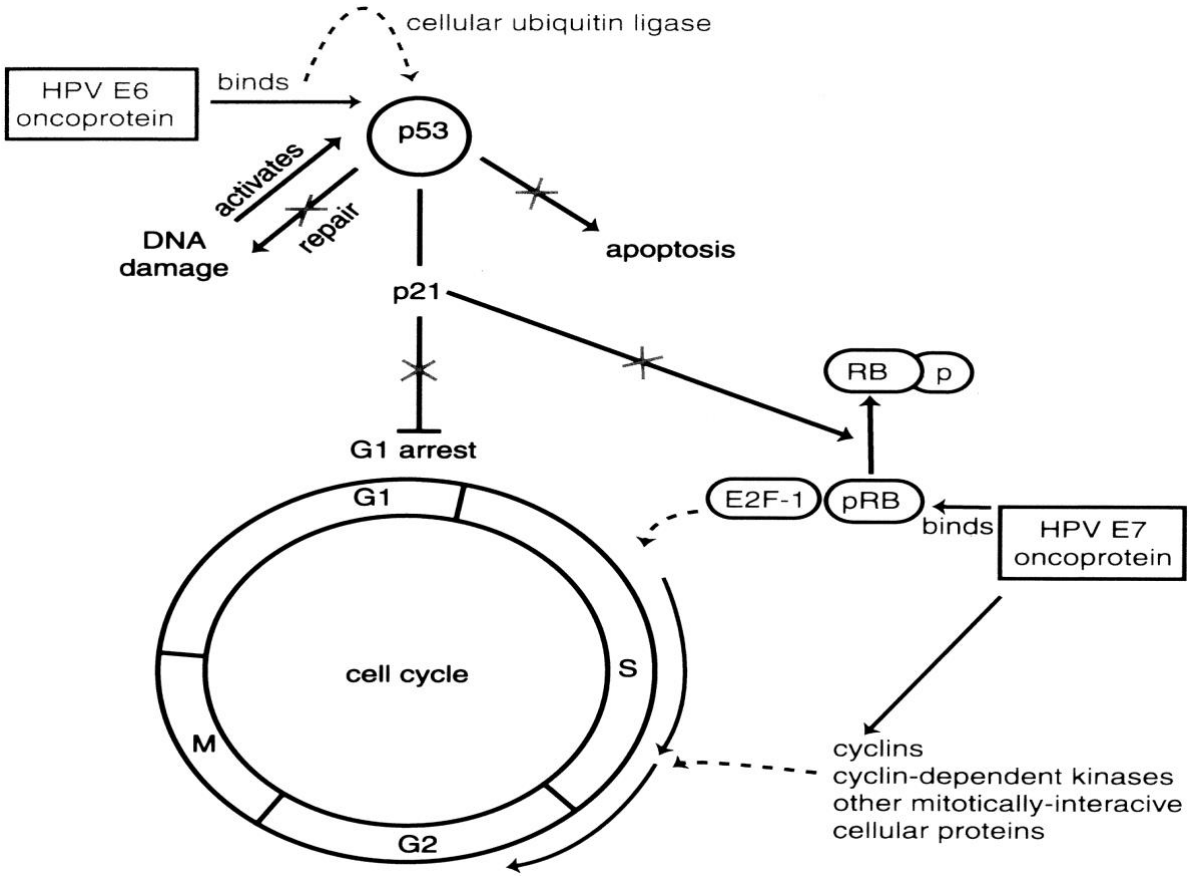
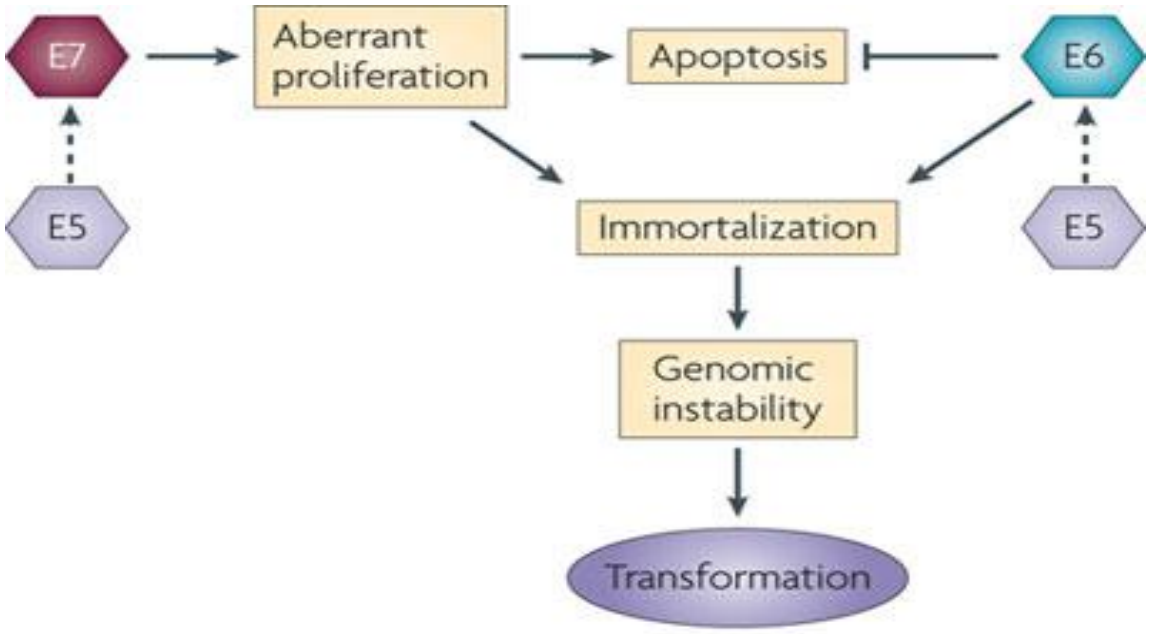


Figure 9: Stages of HPV-associated oncogenesis (Moody and Laimins, 2010).



1.2.6 Immune response in HPV-associated malignancy

The role of both innate and adaptive immunity in the development of HPV-associated HNSCC is poorly understood. Evidence gained from uterine cervix cancer trials would suggest an impeded immune response to HPV is a requirement before malignant change can arise (Woodman *et al.*, 2007). Further evidence to support this comes from studies on immunocompromised patients (HIV positive) who demonstrate an increased frequency, duration and pattern of HPV infection in comparison to normal controls (Schiffman *et al.*, 2007).

It is estimated that <1% of high risk HPV infections within the uterine cervix progress to carcinoma *in situ* (Tindle, 2002). Within the oral cavity, the exact mechanism behind persistence of HPV infection and subsequent risk of oncogenic transformation are likely to involve multiple factors e.g. local tissue environment (Waldeyers ring), virus clearance rate and exposure to tobacco / alcohol (Gillison *et al.*, 2012).

HPV can avoid detection by the host immune system by restricting antigen presentation – a process aided significantly by the absence of a haematological phase within the viral life cycle (Stanley, 2012a). Immunogenic peptides from both E6 and E7 oncoproteins are not efficiently processed or presented by the host cell during replication, and, this correlates with reduced expression of human leukocyte antigen (Moody and Laimins, 2010). This may be due to a variety of mechanisms including the restriction of E6 and E7 to the cell nucleus or by limiting transcription of the virus before host cell integration (Tindle, 2002).

Regarding the L1 capsid protein, a study investigating the epitope-specific response by Langerhans cells found an ameliorated response to human papillomavirus virus like particles. This leads to the inference that the virus limits expression (and thus detection) of capsid protein within superficial differentiated epithelial cells (Fausch *et al*., 2002).

Innate immune response to a viral infection involves the activation of type 1 interferons which have anti-proliferative, anti-angiogenic and immune-stimulatory activities (Kanodia *et al*., 2007). HPV E6 and E7 have demonstrated ability to impede the function of type 1 interferon-alpha and -beta, which further prevents response by dendritic cells (Stanley, 2012a). E6 down-regulates Signal Transducer and Activator of Transcription 1 (STAT-1) and binds to Interferon Regulatory Transcription Factor (IRF-3) which prevents transcription of interferon-beta mRNA. E7 reduces induction of interferon-alpha via loss of Interferon-Stimulated Gene (ISG) factor-3 transcription complex (Kanodia *et al.*, 2007).

Immune tolerance is a further evasive tactic employed by HPVs. First, E7 shares antigenic motifs with several human proteins and this molecular mimicry can lead to a reduced immune response (Vu *et al*., 2010). Second, a relative lack of inflammatory cytokines induced by high risk HPVs may lead to dendritic cells transmitting a "tolerance" signal to T lymphocyte cells, and subsequent loss of an adaptive response (Tindle, 2002).

In summary, the mechanisms described above all work to reduce the detection and presentation of human papillomavirus antigens, which in turn ameliorate host immunogenic response. It is perhaps surprising to note that the overwhelming majority

of HPV infections resolve naturally after activation of a cell mediated immune response. Therefore, we may speculate that modification of immune responses to HPV-associated HNSCC can potentially influence disease outcome (Rapidis and Wolf, 2009).

1.2.7 Vaccination

Prevention strategies can involve either reducing the risk of exposure to the various infective agents or the development of a screening tool. Regarding the former strategy, a prophylactic vaccine, based on the synthesis of recombinant L1 proteins into virus-like particles (the capsid with no genetic material), is now widely available. Antibodies targeting the major viral capsid protein L1 have demonstrated high levels of efficiency in patients who have no established HPV infection (Riemer *et al.*, 2010). Indeed, several studies demonstrate that L1 vaccine antibody levels and the overall rate of seroconversion are significantly higher when compared to natural HPV exposure (Stanley, 2008).

The Gardasil™ vaccination is now available in the United Kingdom for all girls between the age of 12-13 years. This new vaccine is more comprehensive than Cervarix™ (previously dispensed in the UK) and protects against both low- and high-risk subtypes (6,11,16,18).

Several randomised controlled trials have shown evidence to suggest that either vaccine can decrease the incidence of long term genital HPV16 and HPV18 infections and subsequent progression to uterine cervical intra-epithelial neoplasia. This protection also extends to other genital regions such as the vagina, vulva, anus and penis (Nyitray *et al.*, 2011). As yet, the impact of these vaccines on the incidence of persistent oropharyngeal HPV infection has not been studied (Pickard, 2012).

In 2012, a study from the USA recommended that vaccine trials in men could enhance our knowledge of the efficacy of HPV vaccination, especially considering the higher rates of HPV-positive OPSCC in males compared to females (Gillison *et al.*, 2012). HPV vaccination in males would not only raise the potential of protection against

OPSCC, but also other HPV related cancers of the anogenital region (Masterson and Lechner, 2016). The problem with the herd immunity model (regarding female only HPV vaccination) is that it completely excludes men who have sex with men (MSM), a group that are more likely to be infected with HPV than heterosexual men and have an incidence rate of anal cancer equivalent to that of cervical cancer in an unscreened population.

It has been argued that the increasing frequency of male HPV-associated disease, is now comparable to that in women, and this is reason enough to vaccinate men and stop relying on herd immunity to protect a large proportion of our population (Stanley, 2012b, Masterson *et al*., 2016b). Recent data would suggest that if we started vaccinating boys today, it may be 30-35 years before the benefits are apparent for protection against HPV associated HNSCC (Schache *et al*., 2011).

As previously stated, patients with established viral infection do not benefit from vaccination with L1 capsid proteins. HPV vaccines that focus on antigen-specific T cell mediated immunity against E6 and E7 are now in development, which may confer this additional therapeutic benefit (Riemer *et al*., 2010).

1.3 Epstein-Barr Virus

1.3.1 The virus

Epstein-Barr Virus (EBV) is a member of the herpesvirus family and is also referred to as human herpesvirus-4 (Serraino *et al*., 2005). The virus was first identified in the 1960s by the Epstein laboratory group in a cell line derived from Burkitt's lymphoma. Serological studies indicate that most adults worldwide (>90%) are infected with EBV (Williams and Crawford, 2006). In developed countries, EBV manifests in ~25–75% of cases as infectious mononucleosis (a self-limiting disease characterised by lethargy, sore throat, fever and lymphadenopathy).

The primary infection is transmitted by saliva and mainly gains access to epithelial cells in the tonsil and tongue base region (Waldeyer's ring) (Evans, 1971). After infection of the host cell, which may only require a single virion, the linear genome persist as episomal DNA after binding. The virus is usually asymptomatic or mild in children. Most patients become long-term carriers of EBV, without experiencing disease. The virus persists in two ways: in epithelial cells of the oropharynx / salivary glands / urogenital tract or more commonly in circulating infected immune B cells (Borza and Hutt-Fletcher, 2002). In some patients, the latent virus can cause malignant tumours such as B- and T-cell lymphomas or nasopharyngeal carcinoma (Borza and Hutt-Fletcher, 2002).

1.3.2 Structure and Life Cycle of EBV

The virus primarily proliferates via a non-lytic mechanism (Williams and Crawford, 2006). During this non-lytic latent process, latent membrane proteins (LMPs) and virus-derived nuclear proteins (EBNAs) are expressed by infected host cells (Young and Rickinson, 2004).

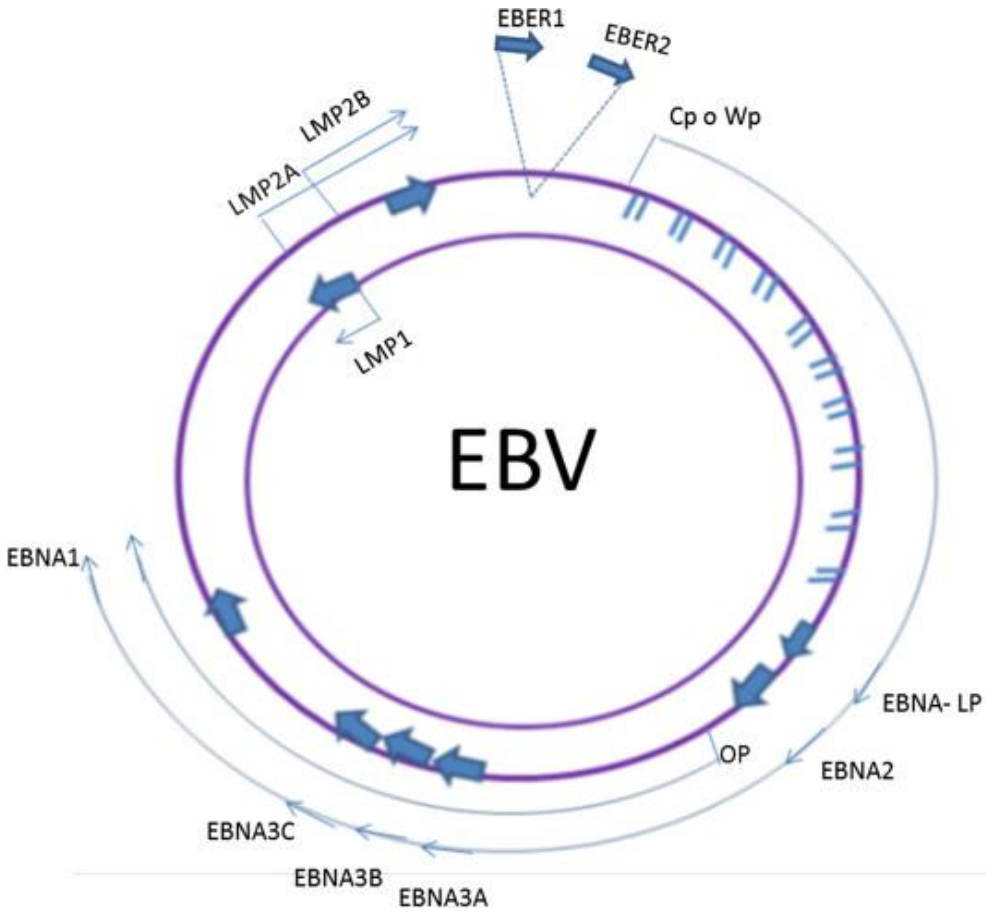
A developing aspect of research into EBV is focused on how viral proteins may play a role in lymphoproliferative disease. Although EBV infections commonly lead to mild symptoms, attempts at treatments with antivirals have generally been unsuccessful (Prabhu and Wilson, 2016). It is particularly difficult to control spread between hosts, as symptom-free carriers continue to transmit the virus years after initial infection.

EBV has a linear, double-stranded DNA genome of approximately 184 kb that is sheltered within a protein capsid (Fuentes-González *et al.*, 2013). The virus encodes 85 genes and open reading frames (ORFs) are divided up into distinct lytic and latent sections that interact with a wide variety of cytokines, signal transducers and anti-apoptotic molecules (Serraino *et al.*, 2005). These products and events promote EBV infection, immortalization and transformation (Young and Rickinson, 2004).

In the latent phase of infection, characterised by no production of EBV virus, the EBV genome exists in a circular form localised in the host cell nucleus (Young and Rickinson, 2004). A minority of genes are expressed which lead to immortalization of the host B cell (Fuentes-González *et al.*, 2013). The expression of the full repertoire of viral latent genes is referred to as Latency III (**Figure 10**).

EBV's success as a pathogen relies on its ability to avoid the host immune system, due to a network of signaling pathways that prevent intracellular and extracellular detection components of the immune response.

Figure 10: Genomic organisation of latent Epstein-Barr Virus. Location and transcription of the EBV latent genes on the viral DNA episome. The arrows at the bottom are coding exons for each of the latent proteins; the latent proteins include three latent membrane proteins (LMPs 1, 2A, 2B) and the six nuclear antigens (EBNAs 1, 2, 3A, 3Band 3C, and EBNA-LP). EBNAs are transcribed from either the Wp or Cp promoter. The top two arrows represent the highly transcribed RNAs EBER1 and EBER2 (non-polyadenylated); their activation is a reliable feature of latent EBV infection (Fuentes-González *et al*., 2013).



1.3.3 EBV–Host Interactions

EBV requires a host cell in which to replicate. Penetration and attachment to this host cell are critical events in viral infection. The lytic phase of the virus is characterised by production of replication of the virus with production of structural proteins, envelope glycoproteins and transactivating proteins.

Like human papillomavirus, cell-mediated immunity (CD8+ / CD4+ T-cell response) plays a significant role in regulating both the primary EBV infection and chronic latent EBV infection (Prabhu and Wilson, 2016).

EBV infection commonly results in the production of antibodies to four separate antigen complexes: EBV-induced early antigen, EBV-induced nuclear antigen (EBNA); EBV-induced membrane antigen; and viral capsid antigen. Antibodies to membrane and early antigens are produced <3-4 weeks, antibodies to EBNA develop late and can persist throughout life (Williams and Crawford, 2006).

EBV types 1 and 2 are the major subgroups identified. EBV type 1 is more common in developed countries and type 2 is more common in homosexual males and sub-Saharan Africa (Young and Rickinson, 2004).

1.3.4 Carcinogenesis of nasopharyngeal carcinoma

EBV is classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) (Prabhu and Wilson, 2016). In EBV immortalized B cells, multiple gene products are expressed during tumour initiation; most are associated with inducing genomic instability, cell proliferation and blockage of apoptosis (Williams and Crawford, 2006). This is turn contributes to cell growth and continued tumour maintenance.

Nasopharyngeal carcinoma (NPC) commonly arises in epithelial cells at the fossa of Rosenmüller, a region of the nasopharynx rich in lymphoreticular tissue, but it can also arise from the roof of the nasopharynx (Young and Rickinson, 2004).

NPC has a remarkable geographic and racial distribution with the highest incidence seen in the South Chinese and Taiwanese populations (Chien *et al*., 2001). NPC is caused by an interplay of viral, environmental and genetic risk factors. Well-established risk factors for NPC include salt-preserved fish and elevated antibody titres against EBV. Specific human leukocyte antigen class I genotypes are also associated with this disease (Chang and Adami, 2006).

EBV is commonly detected in nasopharyngeal carcinomas. Serological investigations have demonstrated elevated levels of IgA and IgG antibodies against EBV early antigen, viral capsid antigen and nuclear antigens in patients with nasopharyngeal carcinoma (Chang and Adami, 2006). EBV DNA analysis suggests a clonal type (a single EBV infected cell leading to cellular growth,) indicating an early, possibly aetiological, event in the development of nasopharyngeal carcinoma (Pathmanathan *et al*., 1995).

Presenting symptoms for NPC can include unilateral or bilateral nasal obstruction, blood tinged nasal discharge or severe epistaxis, unilateral conductive hearing loss due to Eustachian tube blockage, cranial nerve palsies (particularly III, IV, V, VI, IX and X). However, the most common presentation is a persistent enlarged cervical node. In addition to undifferentiated NPC (most common), the three main subtypes are well, moderately or poorly differentiated keratinizing squamous cell carcinoma (Young and Rickinson, 2004).

The proximity of the nasopharyngeal subsite to the eustachian tube orifice provides the potential for migration of NPC through to the middle ear cavity. Given the established link between NPC and EBV, we also investigated the latter in patients with squamous cell carcinoma affecting the temporal bone region.

1.4 Project hypothesis

What is the principle research aim?

We aim to test the hypothesis that dysplastic epithelial changes preceding the development of HPV-associated OPSCC can be identified by specific molecular and genetic abnormalities. If proven to be the case, this may suggest a role for screening, similar to the uterine cervix model of disease.

What are the secondary research aims?

1. To evaluate the prognostic / therapeutic value of molecular and genetic markers in HPV-associated pre-malignancy / malignancy.

2. To investigate immunological response to treatment in HPV16 positive OPSCC.

3. Do certain HNSCC in less usual sites have a different virological profile? As a specific test of this, SCC within the temporal bone will be investigated for evidence of HPV16 and Epstein-Barr Virus.

Chapter 2

Materials & Methods

2.1 Sample collection

Table 4 Solutions and reagents

Materials	Supplier/Constituents	Catalogue number/Source
1x TAE running buffer	4.8 g/L Tris, 1.1 ml/L glacial acetic acid, 2 ml/L 0.5 M EDTA, pH 8.0	
6x DNA sample buffer	0.5% Orange G, 30% glycerol OR 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol	
1 kb ladder	Invitrogen	15615-024
Agarose (electrophoresis grade)	Biogene	300-200
Ethidium bromide	Sigma-Aldrich	E1510
DNA transfer buffer (20x SSC)	88.23 g/L Tri-sodium citrate, 175.32 g/L NaCl, pH 7-8	
DNase-free RNaseA	Roche Applied Science	11119915001
Lysis buffer	10 mM Tris-Cl (pH 8), 10 mM EDTA (pH 8), 150 mM NaCl, 0.4% SDS	
Neutralisation buffer	NaCl, 87.66 g/L, Tris base 60.5 g/L, pH 7.5	
Phenol [pH 8 equilibrated]	USB Corporation	75829
Proteinase K recombinant	Roche Applied Science	03115879001
Qiagen Plasmid Midi Kit	QIAGEN	28004
QIAprep spin miniprep kit	QIAGEN	27104
QIAquick gel extraction kit	QIAGEN	28704
TE buffer, pH 8	10 mM Tris-Cl (pH 8), 1 mM EDTA (pH 8)	
TRIzol reagent	Invitrogen	15596-018
RNase-free 1.5 ml microcentrifuge tubes	Ambion	12400
Nuclease-free dH_2O, autoclaved, 0.2 μm filtered	Ambion	9937
Purelink FFPE RNA kit	Invitrogen	K156002

2.1.1 Patient samples

Clinical samples were obtained from patients attending the Department of Ear, Nose & Throat, Cambridge University Hospitals NHS Trust, UK. All patients gave written informed consent for this study and ethical approval was obtained from the National Research Ethics Service Committee of East of England (12/EE/44). All experiments were performed in the Department of Pathology at the University of Cambridge.

In patients with oropharyngeal carcinoma, prospective paired fresh samples (normal tissue paired with adjacent malignant tissue after histopathological review) were frozen in liquid nitrogen until RNA / DNA extraction. In addition, prospective blood samples were obtained for a further cohort of OPSCC patients before and after curative treatment (radiotherapy +/- chemotherapy) to allow evaluation of cell mediated immune response to HPV16 E2, E6 & E7. A retrospective cohort of patients with temporal bone and oropharyngeal carcinoma allowed further samples from FFPE blocks.

2.1.2 Patient outcomes

The main outcome for this research was DFS (disease free survival) which equates to time after treatment until first malignant disease recurrence or death. Overall mortality was also investigated. The average prospective follow up period for patients with OPSCC was 43.7 months. The average retrospective follow up period for patients with SCC of the temporal bone ranged from 5-30 years. In chapter three (Investigating HPV and cellular changes in benign, pre-malignant and malignant oropharyngeal disease), the main secondary outcomes included analysis of survival stratified by HPV16 and SYCP2. In chapter four (Investigation of CD4+ & CD8+ cell response to HPV E2, E6 and E7 in patients with OPSCC), the main secondary outcomes included cell-mediated immune responses to HPV16 E2, E6 and E7 in peripheral blood using IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT). In Chapter five (Investigating the role of human papillomavirus and Epstein Barr

virus in squamous cell carcinoma of the temporal bone) the main secondary outcome included analysis of HPV16, EBV and TP53 status.

2.1.3 DNA extraction

Oropharyngeal carcinoma samples (fresh frozen)

As per our unit protocol (Winder *et al.*, 2009), ~25mg minced tissue samples were placed into a lysis tube containing 250µl digestion mix (10mM Tris, pH 7.5; 10mM EDTA; 0.5% SDS; 200µg/ml Proteinase K) and disrupted on a bench vortex for 20 minutes and then incubated overnight at 37°C. Proteinase K inactivation involved incubation at 56°C for 10 minutes, then the lysate was subjected to a choloroform / phenol extraction: equal volumes (250µl) of chloroform and phenol were added, the mixture was allowed to stand for 10 minutes and then centrifuged for 15 minutes (13,000 rpm, 4°C); the top aqueous, transparent layer was collected and the remaining contents discarded. DNA was precipitated with 1ml 100% cold ethanol overnight at -20°C. The DNA pellet was obtained by centrifugation for 15 minutes (8,000 rpm, 4°C), and the supernatant discarded. The pellet was air dried and re-suspended in 200 µl PBS. RNase digestion and total genomic DNA isolation (column extraction) were then performed using the DNeasy Blood and Tissue Kit (QIAGEN Ltd, UK), according to the manufacturer's instructions. DNA was eluted with 50µl milliQ H_2O (deionised double-distilled) and stored at -20°C until quantification. DNA concentration and purity was ascertained by use of a Nanodrop™ 1000 spectrophotometer (LabTech International) and associated software. All samples had a 260/280 nm absorbance ratio between 1.8 and 2.2 and were diluted in milliQ H_2O to ~2.5ng/µl prior to PCR.

Temporal bone carcinoma samples (FFPE)

Samples underwent a de-calcification process (Department of Histopathology, Cambridge University Hospital NHS Trust, UK) with trichloroacetic acid (Callis and Sterchi, 1998) and were stored at -70°C for a variable period. A consultant histopathologist with expertise in head and neck pathology reviewed each tissue block

to ensure adequate tumour sampling. Total genomic DNA was extracted from FFPE samples (9 x 3.5 μm sections) using the QIAamp tissue kit, (QIAGEN Ltd, UK) according to the manufacturer instructions. DNA was eluted in purified H_2O (autoclaved + nuclease free) and stored at -20°C until further evaluation. Purity and concentration of DNA was ascertained by use of spectrophotometry. All samples had a 260/280 nm absorbance ratio in the range 1.7-2.2 and were diluted in H_2O to a concentration of 1-27 ng/μl prior to PCR.

Oropharyngeal carcinoma samples (FFPE)

Genomic DNA was extracted from 9 x 3.5 μm sections using a QIAamp tissue kit in accordance with manufacturer's guidelines (Qiagen). DNA was eluted in autoclaved and nuclease-free H_2O and stored at -20°C. Concentration and purity of DNA was assessed by spectrophotometry. Samples with an absorbance ratio (260 / 280 nm) in the range of 1.8–2.2 were diluted with H_2O to a concentration of 1– 25 ng / μl prior to PCR.

2.1.4 RNA extraction

Oropharyngeal carcinoma samples (fresh frozen)

RNA was extracted from fresh tissue using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 1 ml of TRIzol reagent was used to lyse cells isolated from ~25mg fresh tissue (in a bullet blender at speed 7 for 5 minutes). The lysate was then transferred to an RNase-free 1.5ml microcentrifuge tube, and stored for 5 minutes at room temperature to permit the dissociation of nucleoprotein complexes. Chloroform (200μl) was added to the TRIzol reagent (1ml) and the tube vigorously shaken for 15 seconds. Following a 3-minute room temperature incubation, the tube was centrifuged at 13,000 rpm for 15 minutes in a chilled (4ºC) centrifuge. The upper aqueous phase, containing the RNA, was then transferred to a microcentrifuge tube (1.5ml), on ice, and 0.5ml isopropanol per 1 ml of TRIzol reagent used added. The

RNA was precipitated for 3 hours at -20ºC before being pelleted for 15 minutes at 13,000 rpm in a chilled, 4ºC, centrifuge. The RNA pellet washed in 1 ml 75% ethanol per 1ml of TRIzol reagent used. The supernatant was carefully removed and the pellet air-dried for 5-10 minutes, avoiding excessive drying which would reduce solubility. The RNA was then re-suspended in ~75-100µl nuclease-free dH2O. The concentration was determined by a spectrophotometer and then stored at -80ºC.

Oropharyngeal carcinoma samples (FFPE)

Using a protocol adapted for use with Laser Capture Microdissection (Meitner, 2011), tissues samples from normal, dysplastic and invasive malignancy were collected into 0.2ml PCR tube with 20µl lysis buffer from an Invitrogen Purelink FFPE RNA kit (18.5µl melting buffer solution + 1.5µl proteinase K,) and stored immediately on dry ice. A further 130µl melting buffer and 8.5µl proteinase K was added to the mixture and incubated at 60°C for 60-180 minutes until the lysis was complete. After transfer to a clean RNase-free Eppendorf tube, the sample was immediately centrifuged at maximum speed (13,000 rpm) for 1 minute and the lysate transferred to a new RNase-free Eppendorf tube (1.7ml).

The binding step from the Purelink FFPE RNA kit involved addition of 200µl Binding Buffer and 400µl 100% ethanol; 800µl of the sample was then transferred to a spin cartridge in a collection tube and centrifuged at 13,000 RPM for 1 minute. The washing step involved addition of 250µl Purelink FFPE RNA kit Wash Buffer to the cartridge and centrifugation at 13,000 rpm for a further 1 minute with discard of the supernatant. This step was repeated twice. The elution step involved preheating an aliquot of the RNase-free Water to 65ºC (~250µl). The spin cartridge was placed in a clean 1.7 ml recovery tube. 50µl of RNase-free water was added to the centre of the cartridge and incubated for 3 minutes, before centrifugation at 13,000 rpm for 1 minute. The recovery tube containing total RNA was stored at –80 °C until further use.

2.1.5 cDNA synthesis

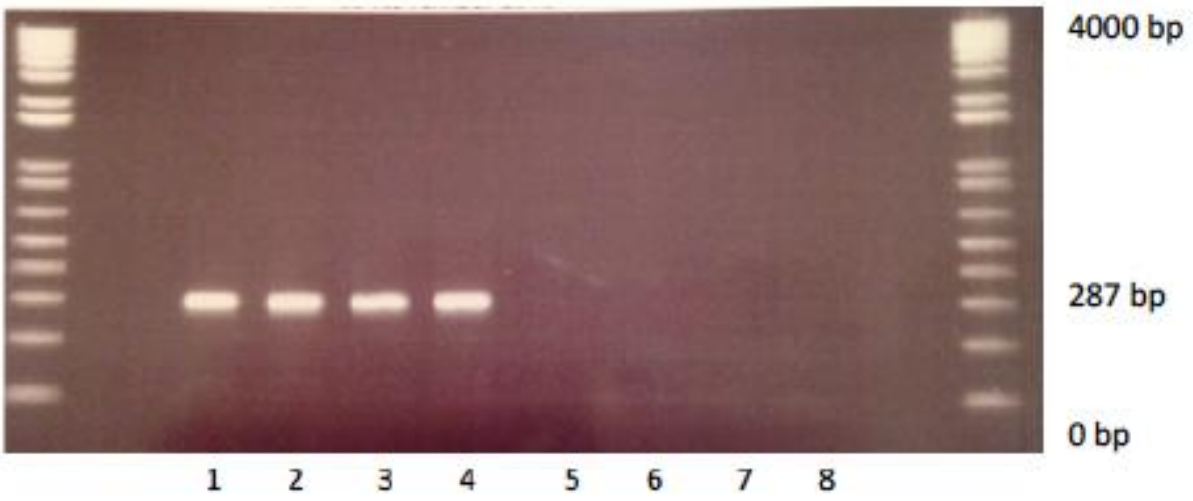
To further investigate HPV16 E6/E7 physical state and LCM extracted tissue, complementary DNA (cDNA) was synthesized from RNA for use in real time quantitative PCR (see section 2.5.4) using a Quantitect reverse transcription kit (QIAGEN) according to the manufacturer's instructions.

To eliminate any contaminating genomic DNA present following RNA extraction (section 2.1.3), reactions were prepared in an RNase-free 1.5 ml microcentrifuge tube (7x Genomic DNA wipeout buffer 2µl; Template RNA up to1µg; RNase free water 14µl final volume) and incubated at 42ºC for 2 minutes before being placed on ice (**Figure 11**).

The reverse transcription reaction consisted of 1µl Quantiscript reverse transcriptase, including RNase inhibitor, 4µl 5x Quantiscript RT buffer, including Mg^{2+} and dNTPs, 1µl RT primer mix (a blend of oligo-dT and random primers), 14µl Genomic DNA elimination reaction (as above) and was incubated at 42ºC for 15-30 minutes. The reaction was then terminated by incubating at 95ºC for 3 minutes. Additional reactions, where no reverse transcriptase enzyme was added (RT negative), were also made and acted as negative controls in downstream applications. Following reverse transcription, reactions were stored at -20ºC.

Figure 11: cDNA synthesis check digest

RT positive lanes (1,2,3,4) show expected PCR product of 287 base pairs. RT negative lanes (5,6,7,8) show no amplification product which implies no genomic DNA contamination of RNA samples.



2.2 Detection of HPV in clinical samples

2.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique used to amplify a segment of DNA to create millions of copies. It is practical and cheap to perform and the concept has multiple applications across a range of biomedical sciences.

Most PCR methods utilise thermal cycling, which involves exposure of reactants to cycles of repeated heating and cooling, DNA melting and enzyme driven DNA replication which proceed rapidly many times in sequence. Selective and repeated amplification is enabled by short DNA fragments (primers) containing sequences that complement the target region, along with DNA polymerase (normally Taq polymerase, which is a heat-stable DNA polymerase enzyme originally isolated from the bacterium *Thermus aquaticus*).

As PCR progresses, the DNA generated is itself used as a template for replication, causing a chain reaction and exponential amplification. DNA polymerase helps to assemble a new DNA strand from free nucleotides, by using single-stranded DNA as a template short primer sequence (as mentioned above) to initiate DNA synthesis. DNA polymerase can only add a nucleotide to the 3'-OH group.

In the first step, DNA melting, the two strands of the DNA double helix are physically separated at a high temperature. The second step is performed at a lower temperature with the two DNA strands becoming template sequences for the DNA polymerase to selectively amplify the target DNA. The precision of PCR results from the use of primers that are complementary to sequence around the DNA region targeted for amplification under specific thermal cycling conditions.

2.2.2 PGMY PCR Analysis

A PCR assay utilizing the PGMY09/11 L1 consensus primer set (**Table 5**) was undertaken. Each sample was amplified with PGMY09/11 primers (final concentration of each oligonucleotide in the primer set was 10 pmol) and β-globin primers (final concentration of each oligonucleotide in the primer set was 2.5 pmol), in the presence of 10x buffer, 4mM magnesium chloride, 25 μm of dNTPs, 1 unit of Platinum Taq DNA polymerase (Invitrogen, USA) per reaction and 4ul DNA sample (Gravitt *et al.*, 2000).

The PCR cycling conditions included a denaturing step for 5 mins at 95°C, followed by 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. This was followed by a final extension for 10 min at 72°C. A 0.8-1.5% agarose gel was then used to confirm the presence of bands specific for both β-globin and HPV.

Electrophoresis grade agarose gels were made by dissolving agarose powder in 1x TAE buffer. The gel solution was then cooled and ethidium bromide added to a final concentration of 0.5μg/ml. The resulting solution was then poured into a tray, using a comb appropriate for the volume and number of samples to be analysed. The gel tray was then placed in a gel tank and submerged in 1x TAE buffer. A 6x sample buffer was added to nucleic acid samples before loading into the wells. In addition, a molecular weight marker (1 kb ladder, Invitrogen) was run to enable the size of the bands to be determined. Electrophoresis was performed at 80-95V for ~60 minutes. Gels were then visualized and documented using an ultra-violet transluminator.

2.2.3 Nested PCR

PCR using the GP5+/GP6+ primers (**Table 5**) was performed as previously described (Winder *et al.*, 2009). The amplification cycle was performed in a reaction volume of 50μl containing 10x PCR buffer, 4mM magnesium chloride, 25μm of dNTPs, 1 unit of Platinum Taq DNA polymerase (Invitrogen, USA), 50 pmol of each primer of the GP5+/GP6+ primer set and 2μl PGMY DNA PCR product. PCR thermal cycling

programme was as follows: denaturing step at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 40°C for 2 min and 72°C for 1.5 min. This was followed by a final extension for 10 min at 72°C. The expected amplicon size was 150 bp.

Positive bands on a gel were excised, the DNA was subsequently purified using QIAquick® gel extraction columns (QIAGEN Ltd, UK) according to manufacturer instructions. DNA purity and concentration were ascertained using spectrophotometry. Samples were then sequenced directly using forward and reverse GP5+/GP6+ primers (Source BioScience, UK). The sequences were then aligned with known HPV types (Basic Local Alignment Search Tool, National Center for Biotechnology Information, Bethesda, USA).

2.2.4 E6/E7 DNA / cDNA PCR analysis

Primers specific for HPV16 E6/E7 PCR assay were utilised as described previously (Sotlar *et al.*, 2004). The PCR cycling conditions were as follows; 94 °C for 5 min denaturing step, followed by 94 °C for 1 min, 60 °C for 45 s and 72 °C for 1 min (x 40 cycles). This was followed by a final extension period of 10 min at 72 °C.

cDNA generated from extracted OPSCC mRNA was subjected to E6/E7 PCR analysis using the same primers and conditions as used for DNA samples (above). Expected amplicon sizes were as follows; unspliced E6-E7 mRNA, 406 bp: E6*I, 224 bp: E6*II, 107 bp. A PCR for GAPDH was performed (amplicon size 122 bp), to confirm that the RNA was suitable for amplification, using primers as described previously (Eagle et al., 2006). The thermal cycling conditions were as follows; 95 °C for 5 min denaturing step, followed by 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min (x40 cycles). This was followed by a final 5 min extension period at 72 °C.

Table 5: PGMY, GP5+/GP6+, HPV16 E6/E7 and GAPDH primer sequences

PGMY09/11 set comprises five forward (11A-E) and thirteen reverse (09F-HMB01) primers (including PC04 and GH20 β-globin as internal controls). The GP5+/GP6+ set consists of a single forward and reverse primer sequence and is a subset of the PGMY amplified region.

Primer set	Primer name	5'-3' sequence
PGMY09/11	PGMY11-A	GCA CAG GGA CAT AAC AAT GG
	PGMY11-B	GCG CAG GGC CAC AAT AAT GG
	PGMY11-C	GCA CAG GGA CAT AAT AAT GG
	PGMY11-D	GCC CAG GGC CAC AAC AAT GG
	PGMY11-E	GCT CAG GGT TTA AAC AAT GG
	PGMY09-F	CGT CCC AAA GGA AAC TGA TC
	PGMY09-G	CGA CCT AAA GGA AAC TGA TC
	PGMY09-H	CGT CCA AAA GGA AAC TGA TC
	PGMY09-I	G CCA AGG GGA AAC TGA TC
	PGMY09-J	CGT CCC AAA GGA TAC TGA TC
	PGMY09-K	CGT CCA AGG GGA TAC TGA TC
	PGMY09-L	CGA CCT AAA GGG AAT TGA TC
	PGMY09-M	CGA CCT AGT GGA AAT TGA TC
	PGMY09-N	CGA CCA AGG GGA TAT TGA TC
	PGMY09-P	G CCC AAC GGA AAC TGA TC
	PGMY09-Q	CGA CCC AAG GGA AAC TGG TC
	PGMY09-R	CGT CCT AAA GGA AAC TGG TC
	HMB01	GCG ACC CAA TGC AAA TTG GT
	PCO4	CAA CTT CAT CCA CGT TCA CC
	GH2O	GAA GAG CCA AGG ACA GGT AC
GP5+/GP6+	GP5+	TTT GTT ACT GTG GTA GAT ACT AC
	GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C
HPV16	E6/E7 (F)	GGG CGT AAC CGA AAT CGG T
	E6/E7 (R)	TCC TCC TCC TCT GAG CTG TC
GAPDH	(F)	CCA CCA TGG AGA AGG CTG GGG CTC A
	(R)	ATC ACG CCA CAG TTT CCC GGA GGG G

2.2.5 Taqman qPCR

Primers and probes utilized in this study were manufactured by Sigma-Aldrich. All reactions were in triplicate and targeted the HPV E6 gene of HPV 16 as well as two internal controls: β-globin and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (de Boer *et al*., 2007, Coleman *et al*., 2008). Details of the primers and probes used are summarised in **Table 6**. Probes were further purified by HPLC. All primers and probe sets were validated using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequence alignment with their target gene was conducted using BLAST Align (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi).

All qPCR reactions were performed in 20µl containing 1x PCR buffer (Qiagen), 4.5mM $MgCl_2$ (Qiagen), 375µM dNTPs (Roche), 100nM primer pairs and probe, 0.5 units (u) Hotstart Platinum Taq (Qiagen) and 4µl template DNA. Standard curves were constructed using purified placental human DNA (Sigma, UK) and purified plasmid containing the HPV16. Serial dilutions of 1 in 5 (10^5 to 6.4pg/µL for DNA or 20,000 to 6.4 copies/µL for HPV 16) were constructed in a triplex. **Figure 12** shows standard curves for all probes.

qPCR amplification was performed using Rotor-Gene 3000 (Corbett Life Sciences). Cycling conditions were as follows: An initial Taq activation and template denaturation step at 95°C for 15 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, with acquisition of fluorescent signal at 60 seconds.

Equation 1: Data was analysed using the Rotor-Gene analysis software (version 6.1) and further analysis was performed using Microsoft Office Excel 2014. The quantification of HPV DNA viral load in samples was accomplished using a standard curve incorporated into each experiment. The quantities of internal control genes were estimated as 'ng/µl' and HPV types were estimated as 'copies/µl'. The number of virus copies within a single cell was calculated using the equation below, the DNA content of a single cell is approximately 7pg DNA (Leyva and Kelley, 1974).

$$Viral\ load\ (copies/cell) = \frac{virus\ copies/\mu l}{cell\ number/\mu l}$$

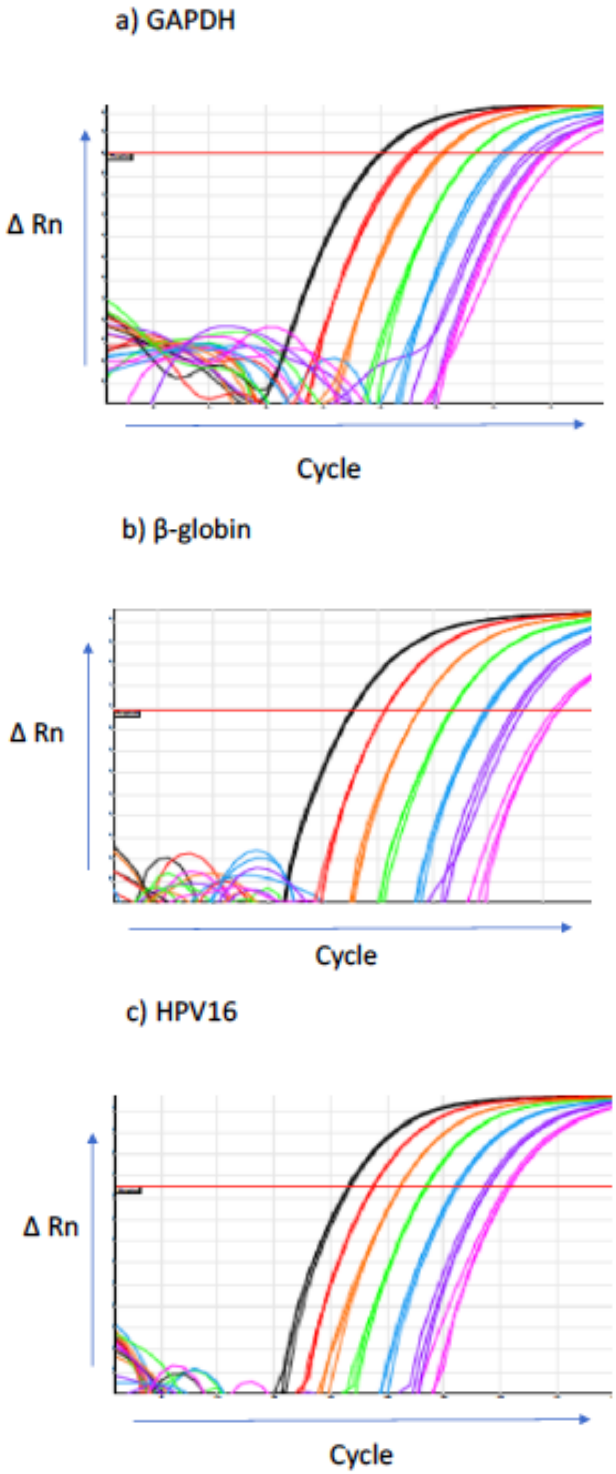
Table 6: Primers and probe sets used in qPCR assay of viral load

These were used in the detection of HPV and Human DNA in samples taken from fresh oropharyngeal samples. Primers and probes were used at a concentration of 100nM. Primer/probe sequences are represented in a 5' - 3' direction. The 5'-fluorescent labels were attached to each probe (6FAM - 6-carboxyfluorescin, Cy5 – cyanine or TET - tetrachloro-6-carboxyfluorescein). Black Hole Quencher 1 (BHQ1) or Black Hole Quencher 3 (BHQ3) were incorporated at the 3' end of each probe.

Target	Accession Number	Forward Primer	Reverse Primer	Probe	Reference
Human β-globin	NG_000007	GACAGGTACGGCTGTCATCA	TAGATGGCTCTGCCCTGACT	[TET]-CTAGGGTTGGCCAATCTACTCCCAG-[BHQ1]	(de Boer et al., 2007)
Human GAPDH	NG_007073	CGGCTACTAGCGGTTTTACG	AAGAAGATGCGGCTGACTGT	[CY5]-CACGTAGCTCAGGCCTCAAGACCT-[BHQ3]	(Coleman et al., 2008)
HPV16 E6	K02718	CCGGACAGAGCCCATTACAAT	ACGTGTGCTTTGTACGCAC	[6FAM]-TGTTGAAGTGTGACTCTACGCTTCGGT-[BHQ1]	(de Boer et al., 2007)

Figure 12: Graphs showing standard curves for all channels

The first dilution of the standard curve was 100ng human placental DNA (a+b) and 100,000 copies HPV 16 plasmid (c). Serial 1 in 5 dilutions were made until the last dilution with 6.4pg DNA and 6.4 copies of HPV 16. The last point on the standard curve was water with no DNA or HPV 16 copies.



2.2.6 EBV PCR

A PCR assay using ~10ng extracted DNA with the EP_5/EM_3 primer set (**Table 7**) was performed as described previously (Markoulatos *et al*., 2001). The PCR cycling conditions were as follows: denaturing step of 94°C for 2 min, followed by 40 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. This was followed by a final extension period of 10 min at 72°C.

2.2.7 TP53 PCR

A 1.84 kB TP53 gene segment, incorporating exons 5–9, was amplified from purified tumor DNA by PCR using primers TP53(F) and TP53(R) (**Table 7**) and sequenced directly (Kusser *et al*., 1993). This region was chosen because somatic mutations outside this DNA-binding region are rare in human malignancies (Oliver *et al.*, 2002). The positive control for TP53 mutation consisted of extracted DNA from the human immortalised cell line K562 (chronic myeloid leukaemia). A negative (Wild Type) control was derived from the CaSki cell line (cervical carcinoma). The PCR cycling conditions were as follows: denaturing step of 95°C for 5 min, followed by 22 cycles of 94°C for 20 seconds, 53°C for 40 seconds and 72°C for 20 seconds. This was followed by a holding cycle at 4°C.

Table 7: Primer sequences for EBV and TP53 PCR.

Primer	**Sequence (5'-3')**	**Virus**	**Genome region amplified**	**Primer position**	**Length of amplimers (bp)**
EP_5 [F]	AACATTGGCAGCAGGT AAGC	EBV	Exon 4/5 terminal protein RNA	775–794	182
EM_3 [R]	ACTTACCAAGTGTCCAT AGGAGC	EBV	Exon 4/5 terminal protein RNA	935–957	182
TP53 [F]	CGTGTTCCAGTTGCTTT ATC	n/a	Exon 5-9	12,985- 14,806	1841
TP53 [R]	AAGAAGAAAACGGCAT TTTG	n/a	Exon 4/5 terminal protein RNA	12,985- 14,806	1841

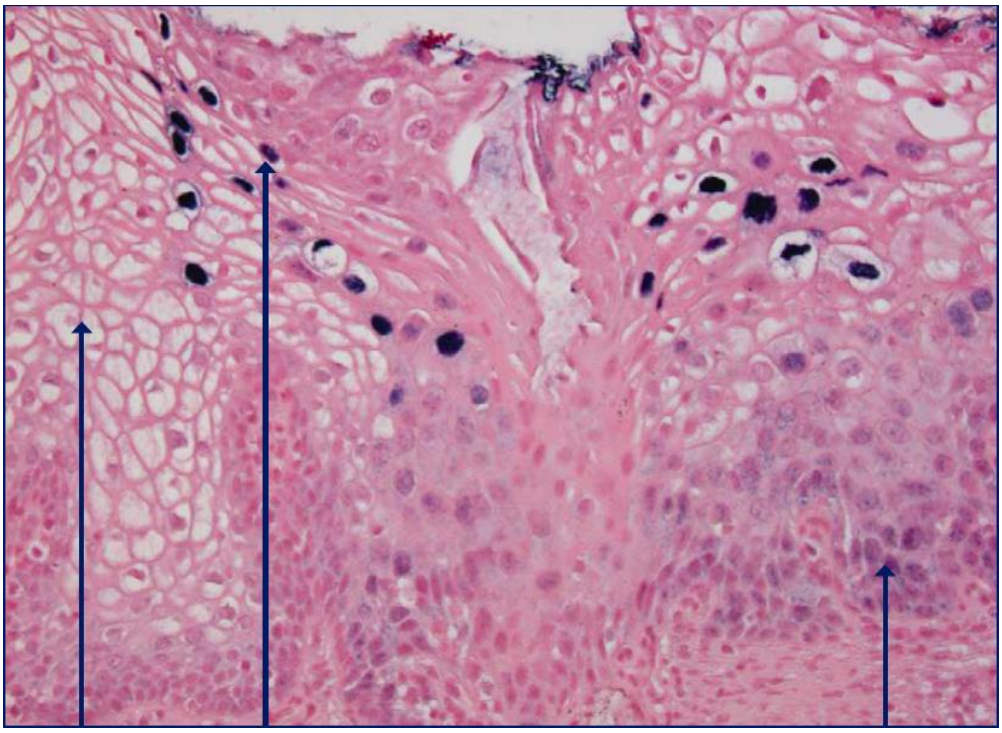
Refers to the position of the first nucleot4156.

2.3 *In situ* hybridisation

In this technique, a labelled strand of DNA or RNA that complements a target DNA or RNA sequence is used to detect the presence of the target sequence in tissue. The probe can be labelled using immunohistochemistry, fluorescence or autoradiography (Jin *et al*., 1997). For this project, digoxigenin (DIG), a steroid, was joined to deoxynucleoside triphosphates and then these labelled nucleosides were incorporated into complementary DNA / RNA sequences. The probes were allowed to conjugate with their target sequences and then excess probe was washed away. To detect probe within tissue, first an antibody (attached to alkaline phosphatase) against DIG was applied. Finally, a solution of nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) was applied. When NBT/BCIP is combined with alkaline phosphatase, an intense, insoluble black-purple precipitate is formed. An example of a HPV DNA positive sample is shown in **Figure 13**.

Figure 13: HPV16 DNA *in situ* hybridization performed on FFPE tissue

The discrete, dot-like nuclear integrative pattern is more often in groups of epithelial cells, located in the basal region of the epithelium.



2.3.1 DNA probes

Escherichia coli bacteria (strain DH1) containing plasmid pBR322 with inserted HPV16 genome was obtained from the Sterling laboratory and grown on 2xTY agar plus ampicillin plates overnight at 37°C while shaking at 220 revolutions per minute (rpm). 50µL of the starter culture was then plated out onto 50mL of 2xTY medium with 50µg/mL ampicillin and allowed to grow overnight (37°C, 220rpm). A single colony was then taken from the plate for midi prep culture (according to the protocol of the Qiagen Plasmid Midi Kit).

Plasmid DNA was linearized by restriction enzyme digest and HPV DNA isolated from plasmid DNA by extraction from gel after electrophoresis. Bam H1 (Roche) produced bands of sizes 7904 and 4361 base pairs (**Figure 14**). 8µg plasmid DNA was digested with 20u of enzyme in 1x buffer B in a total reaction volume of 50µL overnight at 37°C (each enzyme unit [u] is stated to cleave ~1-2 µg of plasmid DNA in 1 hour using these conditions). Digested plasmids were electrophoresed on a 0.8% agarose gel for 70 minutes at 80V (**Figure 15**). The HPV DNA (larger band) was then excised from the gel and purified using QIAquick® gel extraction columns (Qiagen Ltd, UK) according to the manufacturer's instructions.

The linearized DNA was then used to make DIG-labelled probes using the manufacturer's protocol of the DIG DNA Labelling and Detection Kit (Roche), which uses random primer labelling. Finally, probe-labelling efficiency was estimated following the manufacturer's protocol (Roche). In this technique, serial dilutions of probes were made and aliquots placed on a nitrocellulose filter. After cross-linking samples to the filter, a colour change reaction was carried out according to the manufacturer's instructions. Probes labelled as efficiently as the provided control labelled probe (**Figure 16**).

Figure 14: Map of HPV16 in plasmid pBR322

The BamHI sites are indicated at which cutting removes the plasmid backbone from the HPV genome.

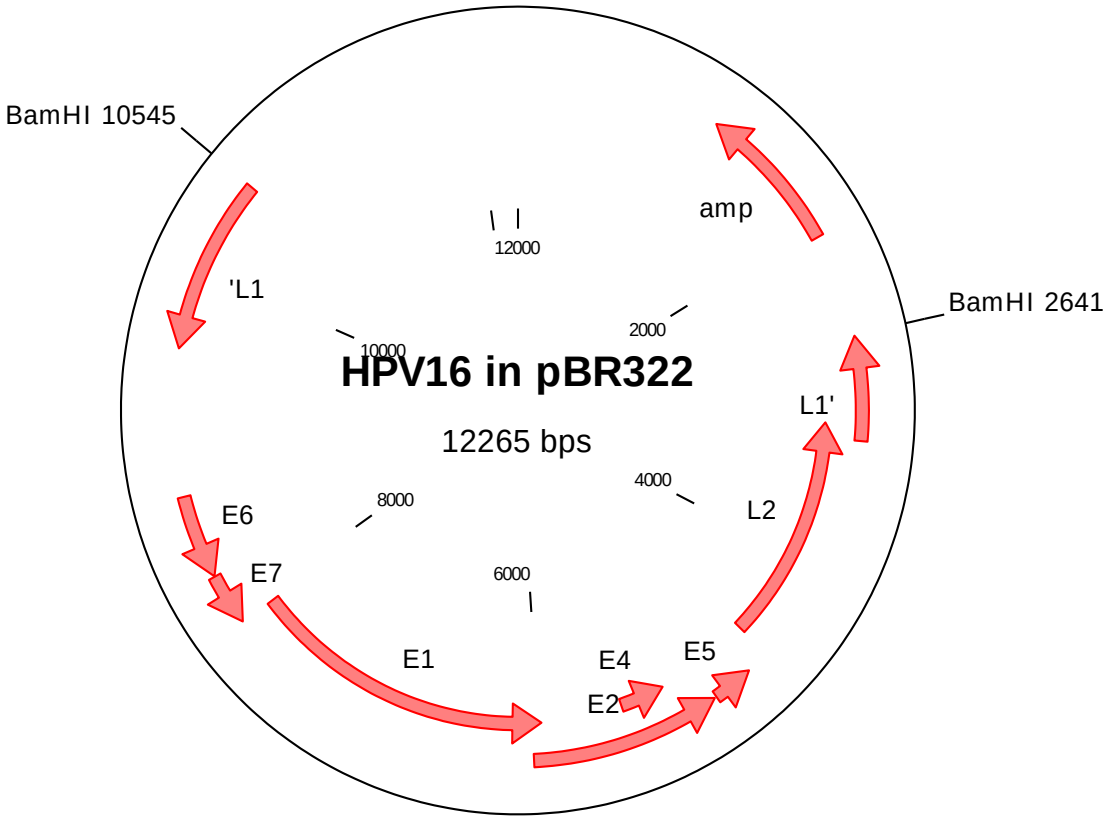


Figure 15: Agarose gel electrophoresis of Bam HI-digested HPV 16-pBR plasmid

Plasmid pBR322 containing HPV16 was cut via a restriction enzyme digest to separate the HPV genome (7.9Kb band) from the plasmid (4.3Kb band). This gel shows the separation of genome from plasmid flanked by a DNA ladder.

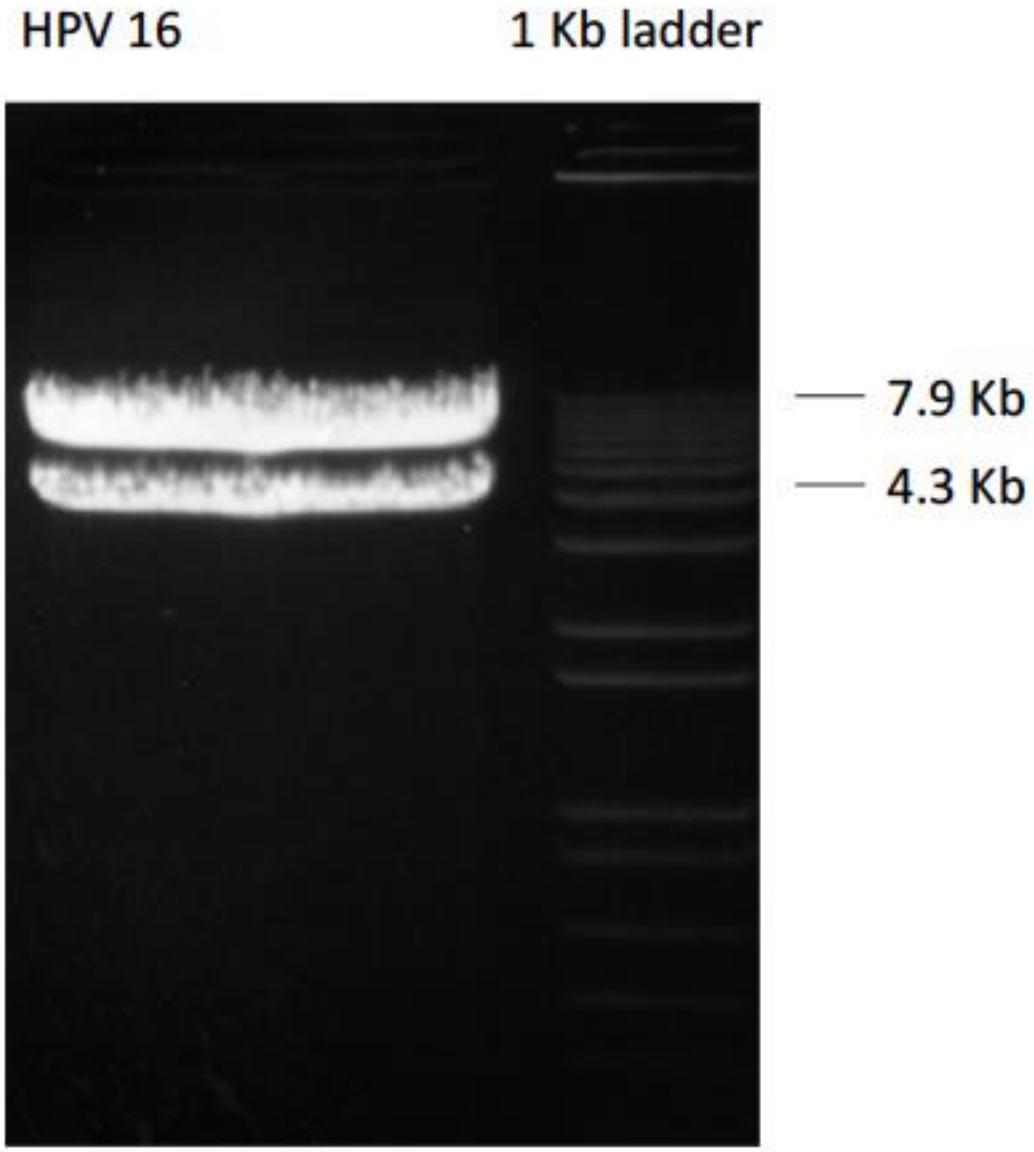
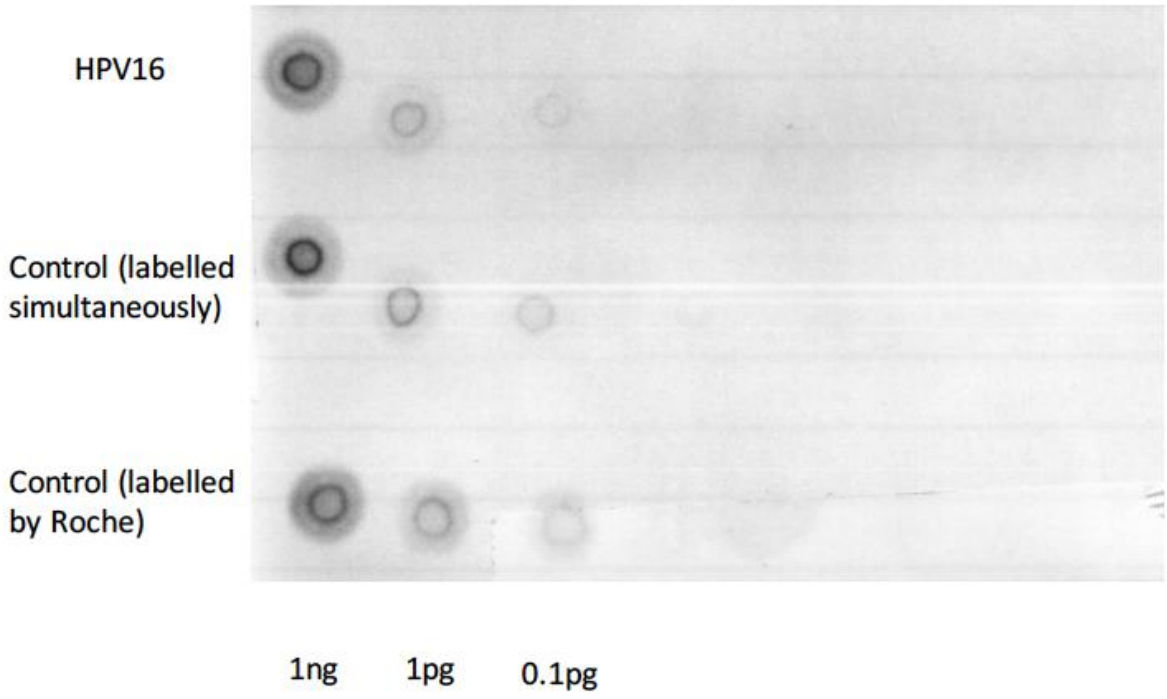


Figure 16: Serial dilutions of HPV 16 DIG-labelled probes

Each serial dilution was spotted onto a nitrocellulose filter. Labelled controls that were either labelled simultaneously with HPV probes or labelled previously and included in the DIG Labelling and Efficiency Kit (Roche) were also diluted and put onto the filter. DIG-labelled HPV probes were labelled as efficiently as control probes.



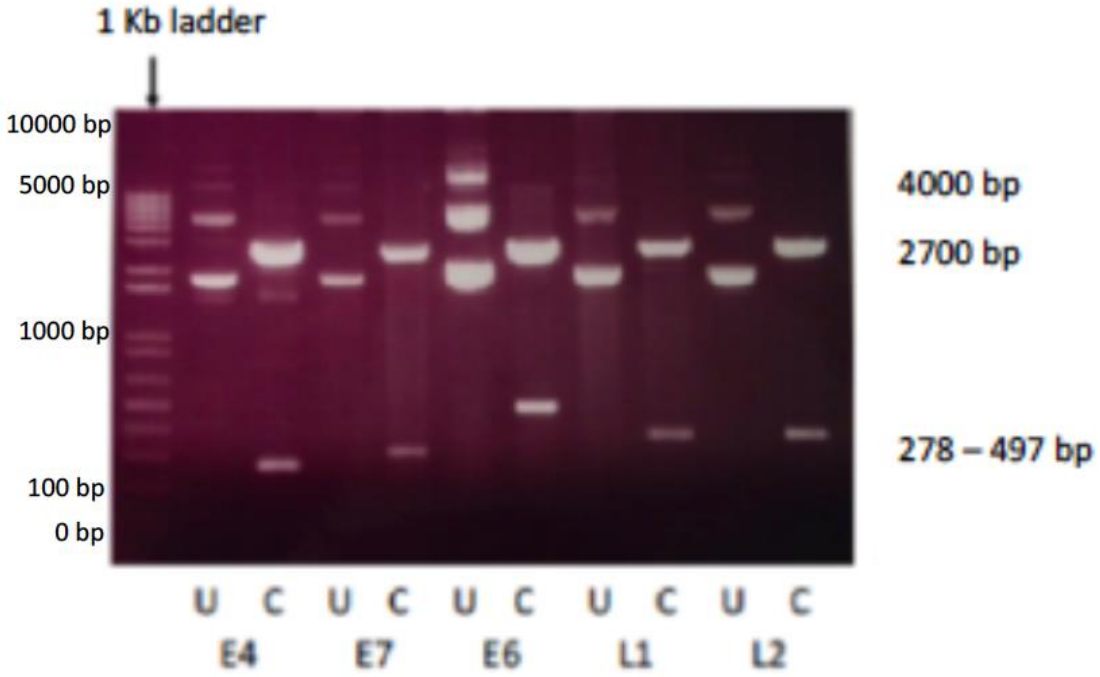
2.3.2 RNA probes

Five probe sequences (HPV type 16 E4, E6, E7, L1 & L2) that had already been ligated into the expression vectors pGEM3Z or pGEM4Z and transformed into bacteria were obtained from the Sterling Laboratory.

Plasmid digests to check for the correct size of the HPV insert were performed for each probe construct using 20u EcoR1 and 20u BamHI in 1x buffer B (Roche) in a total reaction volume of 20µL. Samples were incubated at 37°C for 80 minutes. Samples were run on a 1% agarose gel at 90V for 60 minutes (**Figure 17**).

Figure 17: Plasmid digests for EcoR1 and BamH1

Cut enzyme lanes [C] reveal a large vector (~2700 bp) and small insert fragment which is specific for each probe sequence (Expected HPV fragment sizes after Bam/Eco digestion: E4 = 278bp; E7 = 317bp; E6 = 497bp; L1 = 367bp; L2 = 385bp). [U], uncut sample.



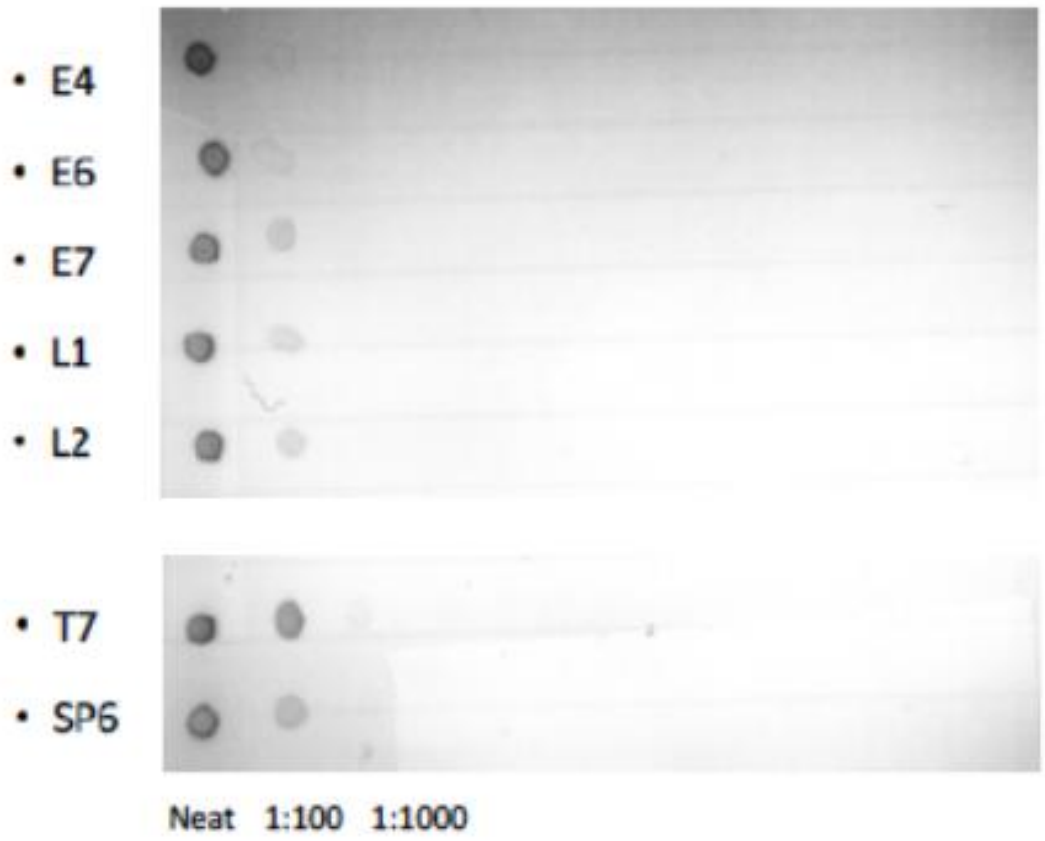
As the check digest revealed fragments of the expected size (**Figure 17**), a subsequent gel extraction was performed. Each sample was diluted to a concentration of 15µg in 50 µL (see below) with the addition of 3µL enzyme (BamHI or EcoR1), according to the plasmid construct and 5µL 1 x buffer B (Roche).

Samples were incubated at 37°C for 80 minutes. Samples were run on a 1% agarose gel at 80V for 60 minutes. Bands were excised from the gel (~2700bp) and DNA purified using the Qiaquick Gel Extraction Kit according to the manufacturer's protocol.

All five probes were labelled according to manufacturer's instructions using the DIG RNA Labelling Kit (SP6/T7) (Roche) and efficiency checked by comparison with control probes (**Figure 18**). The DIG RNA labelling kit labels RNA probes using either the SP6 or T7 polymerase.

Figure 18: Labelling efficiency for RNA probes.

Serial dilutions of HPV16 E4, E6, E7, L1, L2 DIG-labelled probes were made and spotted onto a nitrocellulose filter. Labelled controls for the T7 and SP6 promoter were also diluted and put onto the filter. Probes were not labelled as efficiently as the controls.



2.3.3 DNA *in situ* hybridisation

Pre-hybridisation

FFPE sections were first de-waxed in xylene and rehydrated through an ethanol series before hybridisation with DIG-labelled probes. After rehydration, slides were soaked in 0.02M HCl for 10 minutes, PBS for 5 minutes, 0.01% Triton-X 100 (VWR International) in PBS for 3 minutes, and fresh PBS for another 5 minutes. Slides were then soaked in Proteinase K buffer (5mM EDTA and 50mM Tris, pH 7.5) that had been pre-warmed to 37°C for 10 minutes. Proteinase K was added to the Proteinase K buffer at a concentration of 2.5mg/mL and warmed to 37°C. The resulting solution was added to each slide to completely cover the available tissue, and slides were incubated in a humidified box at 37°C for 10 minutes. Slides were subsequently washed twice with 2mg/mL glycine in PBS for five minutes each. Slides were rinsed in 20% acetic acid in water (4°C) for ~15 seconds. Slides were then soaked in PBS for 10 minutes twice, 4% paraformaldehyde in PBS for 5 minutes, and twice more in PBS for 5 minutes each. Slides were then dehydrated through a series of ethanol dilutions before air drying prior to addition of the probe. DIG-labelled probes were diluted 1:10 in hybridisation buffer (2x SSC, 5% dextran sulphate, 50% deionized formamide, 0.2% Marvel,) and boiled for 1 minute. Probes were then applied to sections and occluded with coverslips. Slides were incubated for 10 minutes in a pre-warmed metal tray at 80°C in order to denature the DNA, and then transferred to a humidified box to incubate overnight at 30°C.

Post-hybridisation

Slides were soaked for up to 10 minutes in 2x SSC to gently dislodge coverslips. Slides were then soaked for 10 minutes in pre-warmed 2x SSC at 50°C (within a polystyrene box to reduce temperature fluctuations). For the washing steps, solutions were placed on a stirrer at 10 minute intervals. Slides were washed in 0.2x SSC at room temperature, pre-warmed 0.2x SSC at 42°C, and then 0.1x SSC at room temperature. Slides were then soaked for 15-30 minutes in Buffer 2 (0.5% blocking

reagent (Roche) in Buffer 1 (0.1M NaCl, 0.1M Tris pH 7.5, 2mM $MgCl_2$, and 0.05% Triton X-100)). Slides were then rinsed briefly in Buffer 1. Anti-digoxigenin-alkaline phosphatase labelled antibody was diluted (1:5000) in Buffer 2. Each sample was then incubated at 30 minutes at room temperature in a humidified box with enough antibody solution to completely cover each tissue section. Slides were subsequently washed in Buffer 1 twice for 15 minutes each. Slides were soaked for 30 minutes in Buffer 3 (0.1M Tris pH 9.5, 50mM $MgCl_2$, 0.1M NaCl) at room temperature. Next, the NBT/BCIP stock solution was diluted 1:50 as per manufacturer instructions (Roche) in Buffer 3. Slides were incubated for 15-30 minutes in the dark with the diluted NBT/BCIP solution. Reactions were stopped by soaking in TE, pH 8.0 (1mM EDTA, 10mM Tris) for 5 minutes. Slides were rinsed in PBS twice, dH_20 once, counterstained in weak, aqueous eosin, and rinsed in dH_20 again. VectaMount AQ was applied to sections and coverslips placed. Pictures were taken with an Olympus BX41 microscope and a QImaging Micropublisher 3.3 RTV camera. Images were captured using Qcapture Pro 5.0 software.

2.3.4 RNA *in situ* hybridisation

Pre-hybridisation

Slides were dewaxed in xylene and rehydrated through a series of alcohols before soaking in PBS for 5 minutes and then fixed in 4% paraformaldehyde for 5 minutes. Slides were washed in PBS 4 times for 2 minutes each and then incubated in pre-warmed Proteinase K buffer (2mM $CaCl_2$, 20mM Tris, pH 7.4,) for 3 minutes at 37°C. Slides were then incubated in a pre-warmed Proteinase K solution (Proteinase K enzyme (25µg/mL) added to Proteinase K buffer) for exactly 10 minutes at 37°C before soaking 4 times in PBS for 2 minutes each prior to soaking in an acetic anhydride (0.25%) / triethanolamine solution (100mM) for 10 minutes. Slides were soaked in PBS 4 times for 2 minutes each. Slides were dehydrated back through an alcohol series and air dried prior to the application of the hybridisation solution.

A DIG-labelled probe was diluted 1:10 in hybridisation buffer (50% deionized formamide, 1x hybridisation buffer (10x SSC, 500mM Tris pH 7.6, 50mM Na_2HPO_4, 50mM NaH_2PO_4, 0.1% Ficoll, 0.1% polyvinyl pyrolidone), 500µg/mL salmon sperm DNA, 250µg/mL tRNA, 2mM DTT, 10 mg/ml). The slides were incubated overnight in a humidified box at 37°C after applying coverslips.

Post-hybridisation

2x SSC (0.1% sodium dodecyl solution (SDS)) was pre-warmed to 55°C to allow gentle removal of slide coverslips. Slides were then washed for 10 minutes in the same solution for 10 minutes at 55°C. RNase (diluted 2µg/mL in 5x SSC) was added to each section and slides were incubated for 15 minutes at 37°C. Slides were washed in pre-warmed 0.1x SSC, 0.04% SDS twice at 65°C for 20 minutes. Next, slides were soaked in Buffer 2 (as described in the DNA ISH post-hybridisation section) for 30 minutes. Anti-digoxigenin-alkaline phosphatase labelled antibody was diluted 1:5000 in Buffer 2. Slides were incubated for 30 minutes at room temperature with the diluted antibody applied to each section. Slides were washed twice in Buffer 1 for 15 minutes each. Slides were soaked in Buffer 3 (0.1M NaCl, 0.1M Tris-HCl pH 9.5, 50mM $MgCl_2$) for 30 minutes. NBT/BCIP stock solution was diluted in Buffer 3 (1:50) and applied to each section and slides were then incubated room at temperature in a humidified box in the dark overnight. The next day, the reaction was stopped by soaking for 5 minutes in 1x TE, pH 8.0 (1mM EDTA, 10mM Tris). Slides were rinsed in PBS twice, dH_20 twice, and counterstained with aqueous eosin prior to mounting with VectaMount AQ (Vector Laboratories). Pictures were obtained as per the DNA post-hybridisation protocol above.

2.4 Immunohistochemisty

Immunohistochemistry (IHC) utilises the detection of antigens using antibodies. Visualizing an antibody-antigen interaction can be achieved in various ways. The most popular method involves an antibody conjugated to an enzyme (such as peroxidase) that can promote a colour-change reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein, to promote an immunofluorescent reaction.

Antibodies against p16 were used in this study. P16 is a cyclin-dependent kinase inhibitor that slows the cell cycle by inactivating phosphorylation of retinoblastoma protein (Rb) by cyclin-dependent kinases. High risk HPV E7 expression causes the upregulation of p16. Specifically, HPV E7 inactivation of Rb can enhance expression of p16, thus allowing this biomarker to act as a good proxy measure for HPV16 E7 oncogene activity (Robinson *et al.*, 2012).

Antibodies against the proliferative markers minichromosome maintenance protein (MCM) 7 and Ki-67 and were also used in this study. MCM proteins contribute towards a pre-replicative cycle that is important for instigating DNA replication. Quiescent cells or well-differentiated cells tend to down-regulate MCM7. Dysplastic and malignant tissue express MCM7 to a variable extent in epithelial cell layers (Blow, 2005). Ki-67 is also a useful biomarker for assessment of proliferation of cells. However, there are some studies which suggest that Ki-67 is not mandatory for successful replication. Also, systemic factors such as nutritional or immune status can alter Ki-67 expression (Schrader *et al.*, 2005).

Epstein-Barr virus (EBV) is firmly associated with nasopharyngeal carcinoma (NPC) as derived by the presence of EBV DNA within the malignant cells for a majority of cases (Zheng *et al.*, 2015). Latent Membrane Protein 1 (LMP1) is thought to be among the most likely EBV proteins involved with oncogenesis and tumour maintenance (Yang *et al.*, 2010).

2.4.1 IHC protocol

Slides were dewaxed in xylene and rehydrated via graded alcohol solutions, then soaked in PBS and dH_20 for five minutes each. Antigen-retrieval was instigated by microwaving slides for 20 minutes on full power in a solution of citric acid (1.8mM) and sodium citrate (8.2mM). After full cooling, slides were placed in PBS for 10 minutes x2. Slides were then placed in 0.3% H_20_2 for exactly 20 minutes before being washed twice in PBS for 10 minutes each. The tissue samples were blocked by incubating with 1% serum (Vector Laboratories) in PBS for 30 minutes at room temperature. The specific serum was derived from the animal in which the biotinylated antibody was normalised. In this case, horse serum was used. Primary antibodies were used at the following concentrations in PBS: p16 1:20 (BD Pharmingen), MCM7 1:200 (Abcam), Ki-67 1:150 and LMP1 1:150 (Dako, Glostrup, Denmark).

Slides were incubated with the primary antibody overnight in a humidified box at 4°C. The samples were then washed twice in PBS for 10 minutes before application of the secondary biotinylated antibody (Vector Laboratories) in a solution of PBS (1:200). Slides were washed twice in PBS for 10 minutes before application of the ABC reagent (Vector Laboratories) at room temperature for 30 minutes.

Slides were subsequently soaked in PBS for 2x 10 minutes. A further colour chain reagent (3,3'-Diaminobenzidine (DAB)) was applied for approximately 25 minutes – 2 hours depending on the primary antibody. The reaction was stopped after a five minute soak in PBS. Slides were counterstained with haematoxylin (Vector Laboratories) and dehydrated through graded alcohol solutions prior to mounting with Vectamount (Vector Laboratories). Pictures were obtained as per the DNA post-hybridisation protocol above.

2.5 Whole transcriptome analysis

The detection and quantification of RNA in a biological sample can be accomplished through whole transcriptome sequencing (WTS), also known as RNA-Seq (RNA sequencing) utilising next generation sequencing platforms.

In defining the continuously shifting cellular transcriptome, WTS provides information on mutations/SNPs, alterations in gene expression, abnormal splicing, gene fusion and modifications after transcription. A whole host of RNA data can be elicited by WTS such as mRNA transcripts, total RNA, tRNA, miRNA and ribosomal profiling. Furthermore, WTS can delineate exon/intron boundaries following verification and/or amendment of 5' and 3' gene boundaries.

Microarrays, which are founded on hybridisation techniques, were previously used for gene expression analysis before the advent of WTS. WTS has overcome the disadvantage of artefacts that arose in microarrays due to cross-hybridisation and the inability to fully quantify low and high expressed genes, which therefore required prior knowledge of the sequence.

Oropharyngeal carcinoma patients in this study were categorised into HPV- or HPV+ using the techniques described earlier in this chapter. In twenty-four patients, multiple fresh frozen biopsy samples were obtained at the time of diagnostic or therapeutic surgery. All biopsies were selected for downstream applications based on their RNA integrity number after histopathological review.

2.5.1 Investigation of RNA integrity

RNA extracted from tumour samples (see 2.1.3) was assessed for purity using RNA 6000 Nano Chips and the Agilent 2100 Bio-analyzer (Agilent Technologies, CA, USA) according to the manufacturer's instructions. This system, which has now superseded agarose gel electrophoresis in the assessment of RNA integrity, utilizes miniature glass chips that contain interconnected reservoirs. The channels between reservoirs are filled with a gel matrix, RNA samples, and a dye concentrate. Sample components are separated by electrophoresis, the fluorescence detected and translated into electropherograms and gel-like images (bands). Only samples which demonstrated no evidence of RNA degradation were used for downstream studies (**Figures 19-21**).

Figure 19: Electropherogram of non-degraded RNA (RIN=7.6) by the Agilent 2100 Bio-analyzer. Tall peaks represent 18S and 28S rRNA (arrowed) which is characteristic of good non-degraded RNA. FU, ladder position; nt, time in seconds.

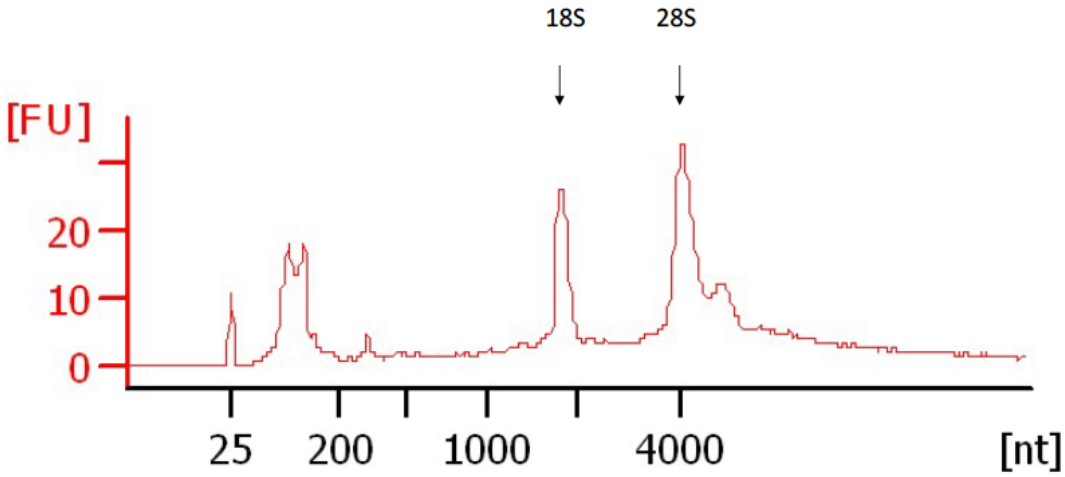


Figure 20: Electropherogram of degraded RNA (RIN=2.8) produced by the Agilent 2100 Bio-analyzer. Note the lack of tall 18S and 28S rRNA peaks. FU, ladder position; nt, time in seconds.

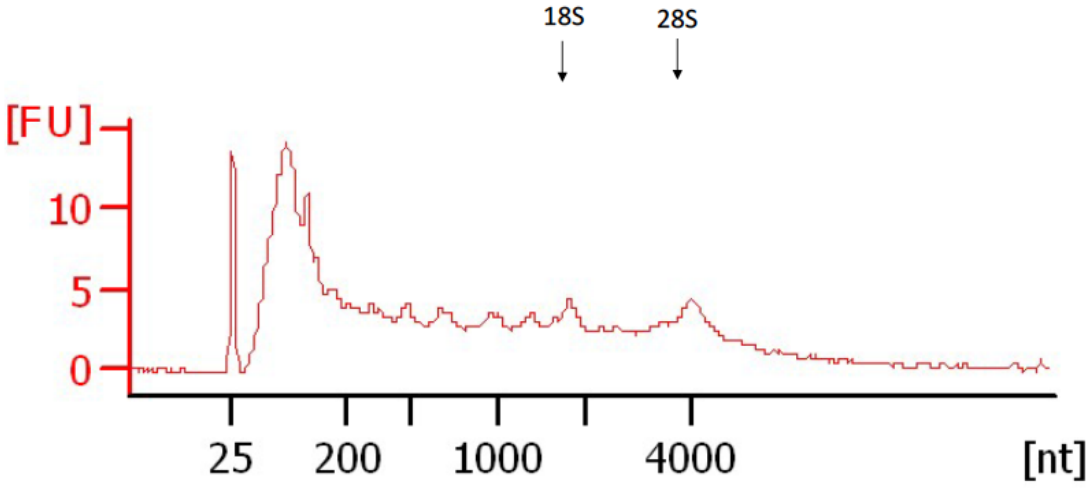
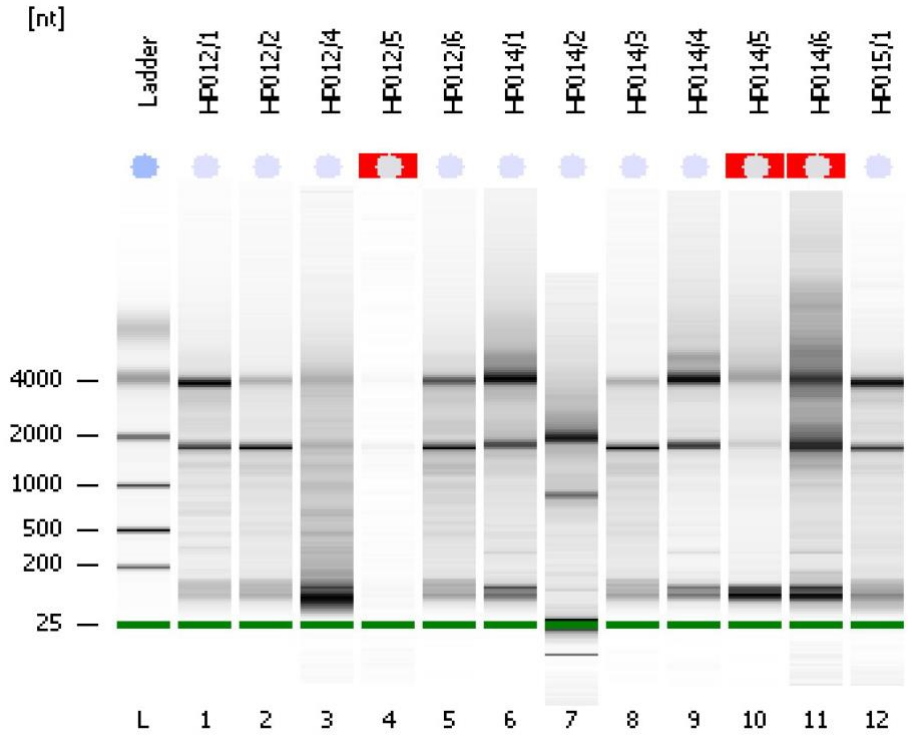


Figure 21: Gel-like images showing prominent bands at 1.8kbp [18S] and 3.9kbp [28S] (see arrows) for the majority of samples



2.5.2 Illumina bead microarray

This study utilised the Illumina Next Generation Sequencing (NGS) platform to provide high-quality gene expression and transcriptome analysis data from a broad range of sample types. This technique can quantify and profile any active gene or transcript, including novel transcripts. In addition, expression microarray technology measures the relative activity of known, predefined genes and transcripts.

All WTS samples were processed by the Cambridge Genomic Service, Department of Pathology, University of Cambridge. In brief, messenger RNA-seq complementary DNA (cDNA) libraries were prepared from ~400 ng of total RNA. Messenger RNA was isolated using poly dT oligonucleotides connected to magnetic beads, fragmented using elevated temperature and divalent cations and converted to cDNA using reverse transcriptase. DNA polymerase I and random primers were then utilized to convert single stranded cDNA into double stranded cDNA. This was blunt end repaired with Klenow DNA polymerase and T4 before adenylation of the 3' end of the fragment. A final purification step utilized gel electrophoresis, with fragments cut out in the range 200 – 300 bp. These fragments were amplified by PCR and sequenced using the Illumina Cluster Station and Genome Analyser (Illumina, Inc, San Diego, CA). Paired-end sequence analysis (51 cycles per end) was conducted with primers specific to the ends of the bridge-amplified cDNA fragments to obtain 51 nucleotides of sequence from each end of all cDNA fragments (Laborde *et al.*, 2012).

Each array on the HumanHT-12 version 4 Expression BeadChip (GPL10558) targets more than 31,000 annotated genes with more than 47,323 oligonucleotide probes derived from the National Centre for Biotechnology Information Reference Sequence (NCBI) Release 38 and other sources. Raw reads from normal epithelium (control) and tumor samples were processed using the Geospiza GeneSifter Analysis Edition pipeline. Expression values for annotated genes were calculated from the aligned data, by adding the number of reads linked to all exons and splicing events for a given gene and dividing that parameter value normalised by the total number of mapped reads in a sample. Two-way analysis of variance identified target sequences with significant differential expression between normal and tumor tissue and further stratified by HPV status (Tusher *et al.*, 2001). The final choice of target transcripts in the study were

based on gene ontology data supplied by the KEGG (Kanehisa *et al*., 2014) oncology pathway network.

2.5.3 Laser Capture Microdissection

The technique of Laser Capture Microdissection (LCM) has been successfully applied for the isolation of single cells (involving the capture of one cell from tissue samples (Meitner, 2011). LCM has been used to study HPV infection in HNSCC (Malhotra *et al*., 2004) and uterine cervical intraepithelial neoplasia tissue (Callegari *et al*., 2014) from fresh and FFPE samples. The accuracy of estimated RNA within the sampled region is expected to be greater when using LCM to dissect smaller areas of tissue (Kalantari *et al.*, 2009) (**Figure 22**).

To validate findings from the prospective WTS samples (see section 2.5), retrospective FFPE oropharyngeal tissue were mounted on PEN (polyethylene naphthalate) membrane slides by the Tissue Bank, Department of Histopathology, Cambridge University Hospitals NHS Trust, UK. An additional section of tissue was sliced onto Biobond-coated Superfrost slides (VWR International, UK) and stained with H&E by the Tissue Bank to exemplify tissue morphology.

Before LCM, PEN membrane slide-mounted tissue sections were dewaxed and rehydrated (1 minute in 95%, 70% ethanol and milliQ H_2O) through an ethanol series before staining very briefly with 0.4% cresyl violet solution. The slides were immediately washed with distilled water to remove any residual staining medium. Sections were allowed to dry prior to transportation on ice.

LCM of fixed tissue sections was performed using Leica LMD7000 Laser Microdissection System (Leica Microsystems Ltd., UK) in the Multi-Imaging Centre, Department of Physiology, Development and Neuroscience, University of Cambridge, UK. A 0.5μm laser beam was used to dissect selected areas of tissue from inverted PEN slides. Dissected tissue was allowed to fall into an underlying collecting tube, containing 40μl PBS (**Figure 23**). The collection tube was stored on ice until RNA extraction (see section 2.1.3). Dissections were performed in triplicate.

Figure 22: Laser capture microdissection

Regions of the oropharyngeal biopsy that represent normal tissue, dysplastic and malignant lesions were identified (A). Using the technique of laser microdissection, a near-IR laser (B) cut around selected areas and liberated the cells away from the tissue to study them.

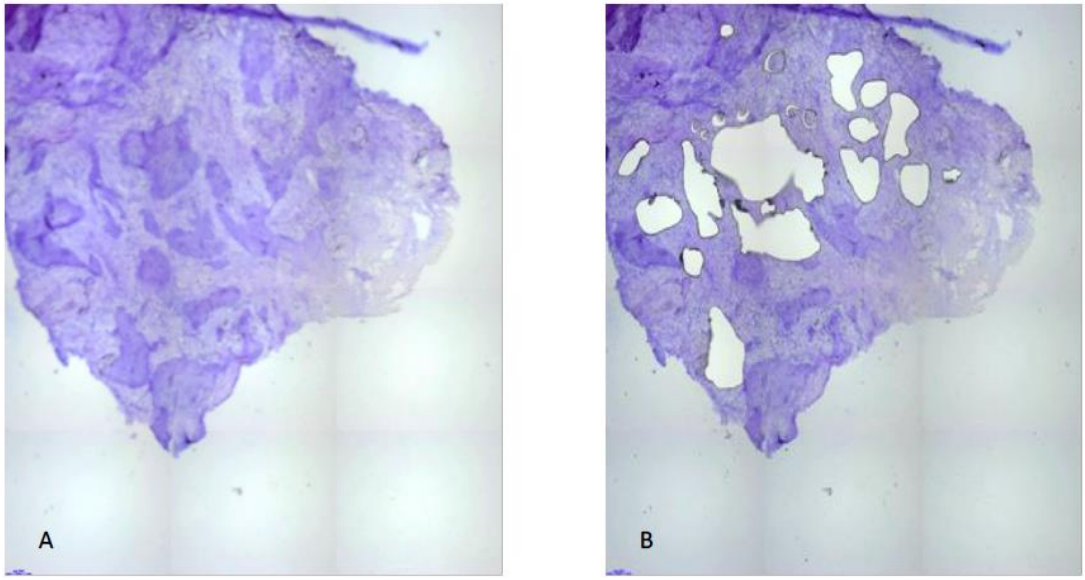
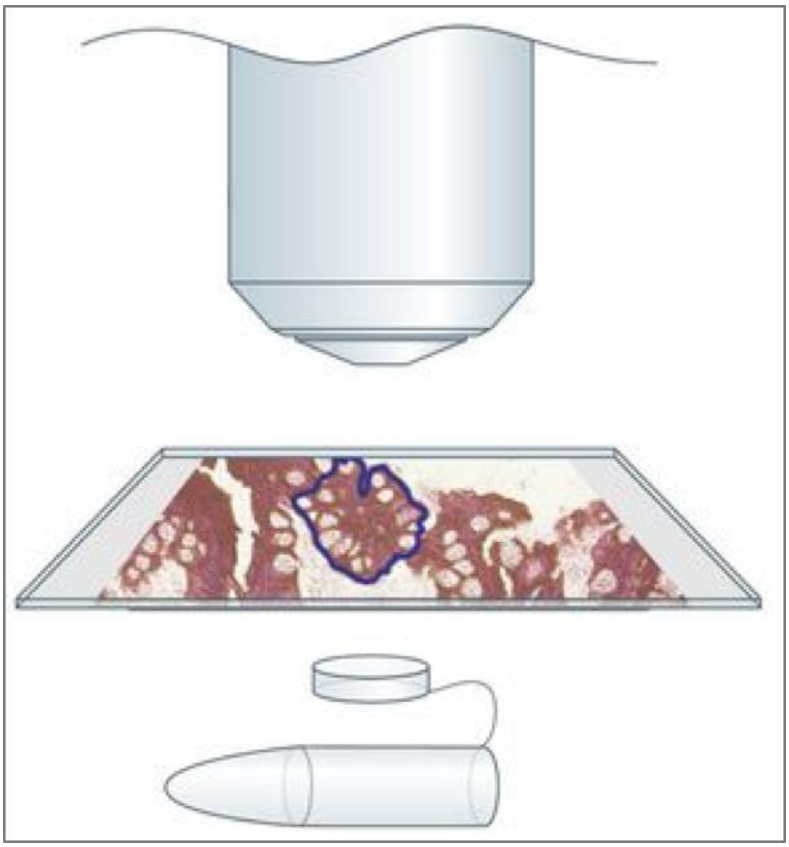


Figure 23: Cell harvest with laser capture microdissection

The Leica microdissection system allows to selectively analyse regions of interest (down to single cells) to obtain results that are specific and reproducible. Proteins and nuclei acid isolated from the dissected specimens can be directed to molecular analyses such as: genotyping, sequencing, PCR and real-time PCR (*Picture courtesy of Leica Microsystems Ltd., UK).*



2.5.4 Real-Time qPCR

As RNA cannot serve as a template for PCR, the first requirement in an RT-qPCR assay is the reverse transcription of the RNA template into cDNA (section 2.1.3), followed by its amplification by PCR. The basic principle of relative gene quantification is to compare the gene of interest (e.g. SYCP2) with that of the internal loading control / reference gene (e.g. β-actin).

Validation of expression data by reverse transcription qPCR analysis utilised the SYBR green method with an Applied Biosystems ViiA™ 7 Fast Real-time PCR system. Primers (Eurofins MWG Operon) were optimized with β-actin as a control gene and then with the transcript region of interest (**Tables 8 & 9**). When the optimal primer concentration produced a linear response to input cDNA concentration (range <1–150 ng), samples were analysed in triplicate for each tested transcript.

For this project, the technique of RT-qPCR had previously been utilised within the Goodfellow laboratory (Division of Virology, Department of Pathology, University of Cambridge). Ideally, the expression of the control gene should remain stable regardless of different experimental conditions. To achieve this, several variables need to be controlled i.e. enzymatic efficiencies, the amount of starting material, and differences between tissues in overall transcriptional activity. Multiple strategies have been applied to reduce these variations.

Table 8: Primers and probe sets used in RT-qPCR assay of WTS selected transcripts from fresh and FFPE oropharyngeal samples. Primers and probes were used at a concentration of 100nM. Primer/probe sequences are represented in a 5' – 3' direction. Primer selection to start <400bp from the 3' end. Amplicon size <200bp. This may optimize amplification of targets with degraded RNA e.g. Laser Capture Micro-dissection.

Gene ID	Illumina ID1	NCBI	Forward	Reverse	Probe	Exon Spanning
SLURP1	ILMN_1705080	NM_020427.2	GGAGGCAGAGTACCCCTTCA	TCAGAGAGGAGGTGCCTGAG	GCTCCTGTGTGGCCACCGAC	Yes
CRNN	ILMN_1727220	NM_016190.2	ACTTCCCCGACTCCAATGTC	GCACCGCAAAGCAGGTAAGA	TGGCTTGTCCTGCATGGCCA	No
SYCP2	ILMN_2095704	NM_014258.2	GGGGGAGGAAGTCCCTAGTT	GTCAAACAGCCTGGTTATGAAA	AGTACTTTCAGCACACTGTTGGAAACA	No
SFRP1	ILMN_2149164	NM_003012.4	GCCAGCAGATACACAGGACA	AGCGATAACATCAAAACATCTGGT	TCCCGTTTCCTCTAGTTTCTTCCTG	No
CCND1	ILMN_1688480	NM_053056.2	CCTTGAGGGACGCTTTGTCT	ACCAGAAATGCACAGACCCA	AGGCTGGTGGCAAGTGCACG	No
CRTC1	ILMN_1803452	NM_019060.2	CTCGGAGTTTGCCCCGTAAA	AAATCACATCGGGGTCAGGG	AAATCACATCGGGGTCAGGG	No
CDKN2A	ILMN_1717714	NM_001195132.1	CCCCCACTACCGTAAATGTCC	CGCAAGAAATGCCCACATGAA	GCACTCACGCCCTAAGCGCA	No
DLG2	ILMN_1676215	NM_001206769.1	GGGAGGGAGGTGGGAATACT	CCGCTGACAACTAGAAACGC	ACACTGCACAGCTTACACTCCTGT	No
ACTB	n/a	NM_001101.3	TCCTCTCCCAAGTCCACACA	AGGGAGACCAAAAGCCTTCA	GGGGAGGTGATAGCATTGCTTTCGT	No
GAPDH	n/a	NM_002046.4	GTAAGACCCCTGGACCACCA	TACATGACAAGGTGCGGCTC	ACTGCTGGGGAGTCCCTGCC	No

Table 9: Primers and probe sets used in RT-qPCR assay of WTS selected transcripts from fresh and FFPE oropharyngeal samples. Primers and probes were used at a concentration of 100nM. Primer/probe sequences are represented in a 5' – 3' direction. Primers were designed to produce an amplicon of approximately 250bp, the site of amplification was within the transcript, but not specifically designed to be at the beginning or end or in the middle.

Gene ID	Illumina ID1	NCBI	Forward	Reverse	Probe	Exon Spanning
SLURP1	ILMN_1705080	NM_020427.2	TTCTGAGCACGGAGCAATGG	TGGTTGAAGGGGTACTCTGC	GACCACGCTGGTGACGGTGG	Yes
CRNN	ILMN_1727220	NM_016190.2	CATCCCACCTGGGCATTAGG	TGGATCGTGGGGTTTCACAA	GCTCACCCGAGGGGAGCTGA	Yes
SYCP2	ILMN_2095704	NM_014258.2	TGTTGCTCGCTAACCAGTCC	TTCCAACTGCTGGAGATCTGG	ACTGGAGCCCAGAGCCTGCT	Yes
SFRP1	ILMN_2149164	NM_003012.4	CTTCCTCTGCTCGCTCTTCG	GACACACCGTTGTGCCTTG	GCCCAATGCCACCGAAGCCT	Yes
CCND1	ILMN_1688480	NM_053056.2	GATCAAGTGTGACCCGGACTG	CCTTGGGGTCCATGTTCTGC	TGCTGGAGTCAAGCCTGCGC	Yes
CRTC1	ILMN_1803452	NM_019060.2	CAGGGTCTGGTTTGTCGTGA	AAAAGCCTTTGGCGGAAACG	GGAGCTCCGCGATGTCCTCT	Yes
CDKN2A	ILMN_1717714	NM_001195132.1	TAGAAGCAGGCATGCGTAGG	AATCGGGGATGTAATGCCAGG	TCGCAAGCTGGCTGGCTCAC	Yes
DLG2	ILMN_1676215	NM_001206769.1	CTGATCAGGGATGCCCACTG	GACTGGCCTGTGCTGGG	GCCCCCACAAGACGAGCACC	Yes
ACTB	n/a	NM_001101.3	GAGCACAGAGCCTCGCCTTT	TCATCATCCATGGTGAGCTGGC	GATCCGCCGCCCGTCCAC	Yes
GAPDH	n/a	NM_002046.4	TCCAAAATCAAGTGGGGCGA	AAATGAGCCCCAGCCTTCTC	CGTCGTGGAGTCCACTGGCG	Yes

The gene quantification method used in this study utilises real-time PCR by comparing the number of cycles required for the fluorescence of a sample to reach a pre-determined level (Pfaffl, 2001). The pre-determined level is known as the cycle threshold (Ct). In this project, the fluorescence threshold was established at a level between the exponential phase and above non-specific background (0.005 relative fluorescence units). Ct values were used to determine relative gene expression as described below: -

Equation 2: *E*ref is the real-time PCR efficiency of a reference gene transcript; *E*target is the real-time PCR efficiency of target gene transcript; ΔCPref = is the crossing point (CP) deviation of control – sample of reference gene transcript; ΔCPtarget is the CP deviation of control – sample of the target gene transcript (**Figure 23**).

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta\text{CPtarget (control – sample)}}}{(E_{\text{ref}})^{\Delta\text{CPref (control – sample)}}}$$

Equation 3: The Pfaffl method of gene quantification requires the calculation of reaction efficiencies. For each gene, a PCR reaction from a normal sample was performed, having made 2-fold serial dilutions covering the range equivalent to 1 – 150ng of cDNA. The Applied Biosystems ViiA™ 7 Fast Real-time PCR system automatically produced a standard curve, and calculated the reaction efficiency using the equation. Where M is the slope of the standard curve which itself is the change in Ct divided the change in log input: -

$$\text{reaction efficiency} = [10^{(-1/m)}] - 1$$

Figure 24: Serial dilution real-time PCR curves

These curves are an example of the data produced by RT-qPCR. In this example, the reference curves (green) represent the fluorescence for the reference gene (β-actin) and the target curves (blue) represent the gene of interest (CDKN2A). From the curves it is evident that the average CP for the reference gene (green) are lower than for the target gene (blue).

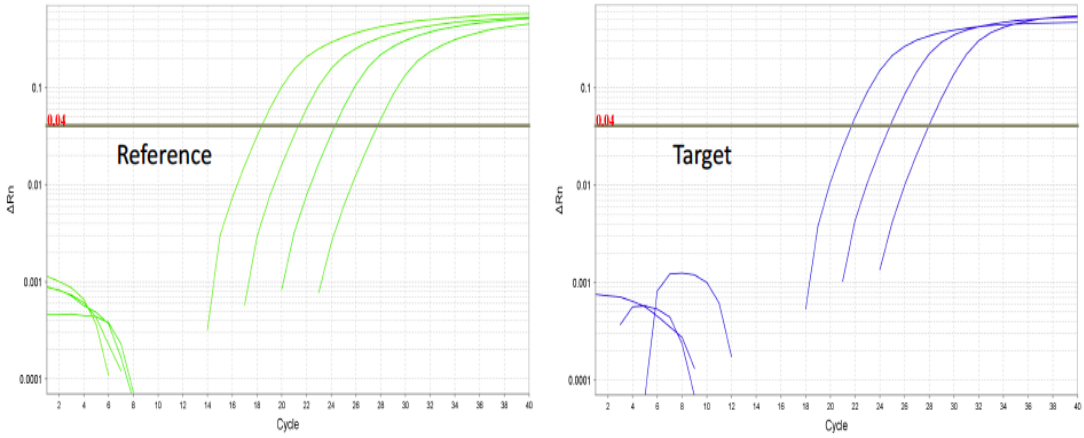
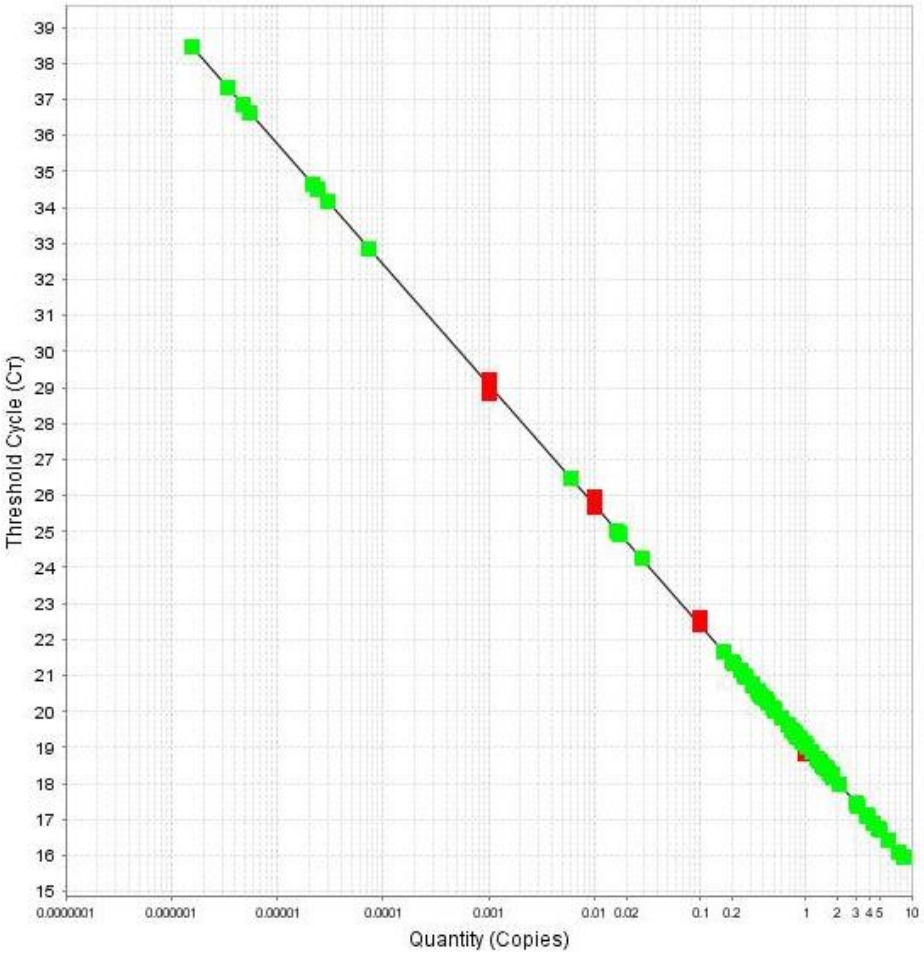


Figure 25: Calculation of standard curves

qPCR data curves for a target gene following repeated serial dilutions allows an automated standard curve calculation. In this example, R2 = 0.998, M = -3.3399, Y-intercept = 19.06; Efficiency = 99.25% (red box = 0.001; 0.01; 0.1 copies).



Standard PCR reactions (total volume 20µl) were performed using 10µl MESA BLUE MasterMix Plus for SYBR® Assay (a combination of Hotstart Meteor Taq together with an inert Blue dye), 0.2µl primer mix (concentration 100nM), 7.8µl dH_2O, cDNA 2µl (concentration 70-80ng/µl). The Applied Biosystems ViiA™ 7 Fast Real-time PCR cycle was performed as follows: -

- Hold stage = 50°C for 2 min followed by 95°C for 10 min;
- PCR stage = 95°C x 15s; 60°C x 1 min (x40 cycles);
- Melt curve stage 95°C x 15s then 60°C for 1 min then 95°C for 15 sec (continuous cycle until end of run)
- Length of run = 1 hour 50 minutes
- Optical emission filters (both PCR & melt curve) = 520nm +/-15; 558nm +/-11; 586nm +/-10; 623nm +/-14; 682nm +/-14

2.6 Enzyme-Linked ImmunoSpot (ELISPOT)

Table 10: Solutions and reagents

Materials	**Supplier/constituents**	**Catalogue number**
Dimethyl sulphoxide (DMSO), liquid	Sigma-Aldrich	D2650
Propidium iodide, 1 mg/ml in H_2O	Sigma-Aldrich	P4864
PI solution	50 μg/ml propidium iodide, 100 μg/ml RNase A	n/a
Foetal calf serum	Bioclear	Batch 122-38507
Penicillin (100 U/ml), streptomycin (0.1 mg/ml), 100x, liquid, sterile-filtered, cell culture tested	Sigma-Aldrich	P0781
Freezing medium	90% FCS, 10% DMSO	
Phosphate buffered saline (PBS)	NaCl (8 g/L), KCl (0.2 g/L), Na_2HPO_4 (1.15 g/L), $K_2H_2PO_4$ (0.2 g/L), autoclaved to sterilise	
Bovine serum albumin	Sigma-Aldrich	A7638

The ELISPOT assay is widely used in human cells and is a mainstay in the diagnosis of tuberculosis and the monitoring of rejection in organ transplantation. In a population of peripheral blood mononuclear cells (PBMCs), the ELISPOT technique can detect infrequent antigen-specific T cell (and B cell) cytokine activity with great accuracy and sensitivity, making it ideal for monitoring cell-mediated immunity.

Furthermore, the IFN-γ (Interferon-gamma) ELISPOT assay can identify and quantify cytokine-producing cells. An individual spot in the assay denotes a single reactive cell. This allows the derivation of quantitative (i.e. frequency of responding cells within the target population) and qualitative (i.e. the specific cytokine or other secreted immune molecule) from the ELISPOT assay.

Requirements for the assay include a 96-well PVDF-membrane microtiter plate within which surfaces are coated with capture antibody binding to the epitope of the specified cytokine. Following seeding during the cell stimulation and incubation phase, a single layer of PBMCs develops along with the target antigen on the surface of the membrane. This antibody-antigen complex will stimulate the T-cell (or B-cell) to release a cytokine(s) which remains on the surface membrane, directly surrounding the secreting cell prior to degradation by proteases, drifting into culture media, or attaching to bystander cells. The retained cytokine can be visualised as an ImmunoSpot i.e. a specific footprint of the activated cell (**Figure 26 & 27**) following further steps.

The advantage of the ELISPOT method over Conventional ELISA assays is its ability to enumerate rare antigen-specific cells by capturing the secreting cytokine which makes it more sensitive and accurate. ELISPOT has theoretical detection limit of 1 in 1,000,000 cells because of the ability to isolate a single antigen positive cell within a population of one million cells. This is an advantage when at a minimum, 100,000 to 500,000 PBMCs is typically used per well for an assay and can go up to one million in the effort to increase the detection of cytokines.

2.6.1 Blood sample collection for ELISPOT assay

A blood sample (40ml) was drawn at baseline after diagnosis and 3 months post treatment. To examine the specificity of the IFN-γ ELISPOT, healthy patients were recruited to the study, each of whom underwent tonsillectomy for a benign condition. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Sweden) and washed with phosphate buffered saline (PBS). The extracted PBMCs were then mixed with fetal calf serum (FCS) supplemented with 10% dimethyl sulfoxide (DMSO; Sigma) before storage in liquid nitrogen.

Pre- and post-therapy samples for each patient were tested in the same experiment. After thawing and washing in cold sterile P10 (PBS + 10% FCS), PBMCs underwent positive depletion of CD56+, CD8+ and CD25+ cells using magnetic microbeads (Miltenyi Biotec, Surrey, UK) according to the manufacturer's instructions. Purity of the mononuclear cell selection (CD4+/CD8+/CD56+) was verified by fluorescence activated cell sorter analysis (see below). 0.5-1.5 x 10^6 CD4+, CD8+ or CD56+ cells were each added to a 15ml conical tube and washed with culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin; Gibco, Paisley, UK).

2.6.2 IFN-γ ELISPOT

To measure IFN-γ release, flat-bottomed 96-well polyvinylidene difluoride (PVDF) membrane backed plates (Millipore, Bedford, UK) were first washed with sterile PBS before coating with 50µl of mouse IgG1 anti-human IFN-γ monoclonal antibody (1-D1K, 15µg/ml in 0.1M NaHCO3 buffer; pH 9.5, Mabtech, Sweden) and incubation at 4°C overnight.

The next day, coated wells were washed x6 with PBS before blocking with 50µl of culture medium (CM) for 3 hours at 37°C. Aliquots of $CD4^+$ / $CD8^+$ / $CD56^+$ cells in CM (100µl ~1 $x10^5$) were added to each well. Stimulatory mAbs to CD28 and CD49d (Pharmingen, Oxford, UK) both at 0.5 µg/ml, were then added for $CD4^+$ T-cell assays (Waldrop *et al*., 1998). Only stimulatory mAbs to CD28 were added for $CD8^+$ T-cell assays (Goon *et al*., 2002).

To induce non-specific cytokine production (required for $CD4^+$ or $CD8^+$ cells), 2µl of phorbol myristate acetate (5ug/mL; Sigma-Aldrich, UK) and 10µl of ionomycin (100µM concentration; Sigma-Aldrich, UK) were added to each positive control well. Peptide libraries spanning the entire length of HPV16 E2, E6 and E7 proteins (Mimotopes, Victoria, Australia) were grouped in pools, and added to the $CD4^+$ / $CD8^+$ cell culture medium to achieve a final concentration of 1 µM for each peptide, prior to incubation for 18 hours at 37°C in 5% CO_2.

The cells were then discarded and the plate washed x6 with PBS/0.05% Tween-20. Each well was then coated with 50µl (4µg/ml) of biotinylated anti-human IFN-antibody (7-B6-1-biotin, Mabtech, Sweden) and the plates incubated for 2 hours at 37°C. A further x6 wash with PBS/0.05% Tween-20 preceded the addition of a streptavidin–alkaline phosphatase conjugate for 1 hour (prepared according to the manufacturer's instructions and added at a volume of 100 µl/well; Biorad, UK). The reaction was terminated by washing with tap water and allowed to air-dry. The number of spots in each well were subsequently counted with digital image software (AutoImmun Diagnostik, Germany).

Figure 26: Schematic diagram outlining the methodology behind an IFN-γ ELISpot assay (Coughlan and Lambe, 2015).

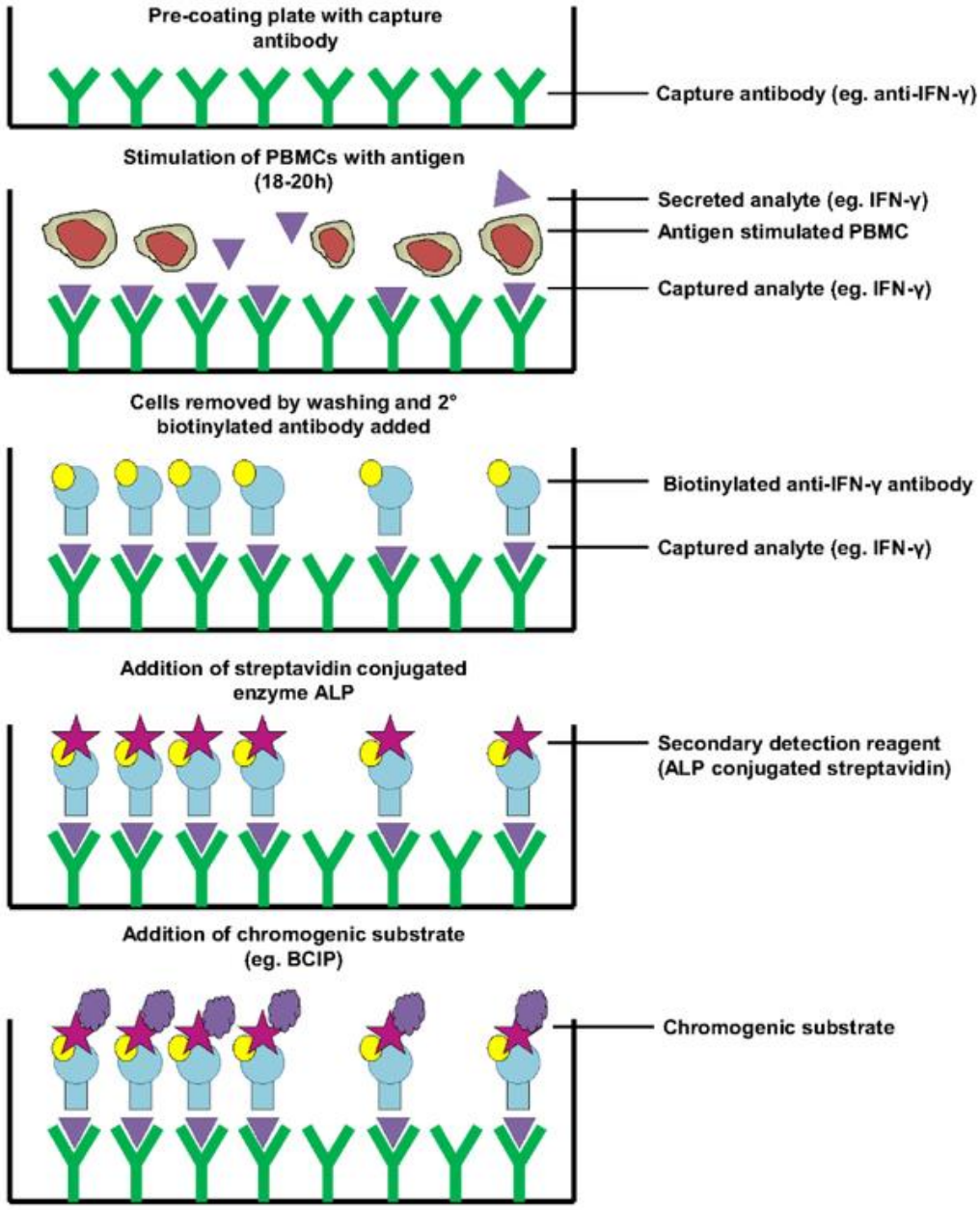
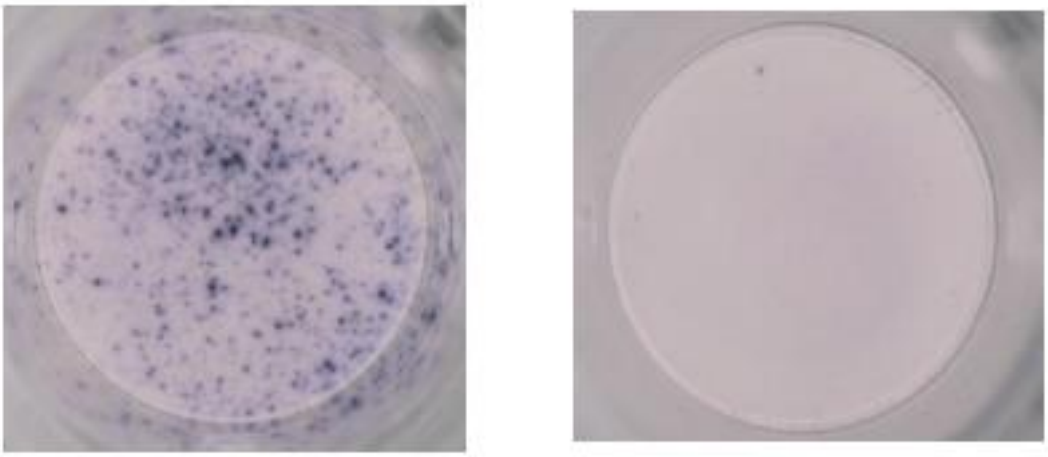


Figure 27: ELISPOT plate demonstrating spot forming cells.



Positive

Negative

2.6.3 Fluorescent activated cell sorting (FACS) analysis

Sub-populations of live or fixed cells can be separated according to fluorescent labeling with this technique. Cells stained with fluorophore-conjugated antibodies can be separated from one another depending on which fluorophore they have been assigned (or alternatively size, shape or granularity of the cell) using forward and side scatter lasers. The fluorophores used in this study included phycoerythrin (PE), Fluorescein isothiocyanate (FITC) and Allophycocyanin (APC). A cell expressing one cell marker is separated using an PE-conjugated antibody that recognizes the marker, and another cell type expressing a different marker is separated using a FITC-conjugated antibody specific for that marker.

Cell for analysis were harvested and pelleted as previously described (see above). The pellet was re-suspended in 1 ml of PBS. A 15 ml Falcon tube was prepared containing 2.5 ml of 100% ethanol. The tube was gently vortexed and the cell suspension added dropwise. The cells were re-centrifuged before being re-suspended in 500µl PI solution (to highlight dead cells; further elucidated later by tryphan blue dye). The tube was then incubated on ice for 15 minutes before being transferred to allow the cells to be fixed at -20ºC overnight or until they were ready to be analysed. The fixed cells were pelleted for 5 minutes at 1000 rpm, the supernatant removed and the pellet washed with 5 ml PBS. Following a 40-minute incubation at 37ºC, 3 ml of PBS was added and the cells centrifuged as above. The supernatant was removed and the cells re-suspended in 500µl PBS, 5% BSA before being passed through a 70µm cell strainer into a 5ml polystyrene round-bottom test tube. The cells were then analysed using a four-laser, 18-colour benchtop flow cytometer (BD™ LSR II, BD Biosciences).

Chapter 3

Investigating HPV and cellular changes in benign, pre-malignant and malignant oropharyngeal disease

3.1 Introduction

OPSCC carcinogenesis may involve progressive transformation from normal epithelium to pre-malignant tissue (dysplasia / carcinoma in situ) to invasive cancer (Miller and Johnstone, 2001, Jayaprakash *et al*., 2011, Leemans *et al*., 2011, van Zeeburg *et al*., 2012, Mooren *et al*., 2014). While the presence of HPV subtypes within invasive oropharyngeal SCC has been evaluated in large epidemiological studies *(*Mehanna *et al*., 2012, Nasman *et al*., 2015) there is limited data on this subject in regions of confirmed dysplasia / carcinoma in situ (Mooren *et al*., 2014).

In a meta-analysis of 269 articles, Mehanna *et al*. (2012) demonstrated that the proportion of HPV+ oropharyngeal carcinoma in North America and Europe has increased over the past ~20 years to ~70%. Nasman *et al*. (2015) found evidence for high risk HPV types in 186/252 OPSCC cases (74%), they also demonstrated a rise in prevalence by comparing samples registered from 2000-2006 to those registered from 2007-2012.

Prior studies in areas of confirmed dysplasia / carcinoma in situ have reported markedly variable HPV prevalence rates due to limitations of size and variable assay techniques (Miller and Johnstone, 2001, Chaudhary *et al*., 2009). Jayaprakash *et al*. (2011) published a meta-analysis of 22 relevant articles, suggesting HPV16 to be present in ~25% of all dysplastic lesions within the oropharyngeal subsite – the same author concluded this to be a conservative estimate due to the inclusion of oral cavity SCC lesions in some of the studies (traditionally a subsite with low HPV16 prevalence).

The majority (~75%) of patients with HPV-positive OPSCC present at an advanced stage (III/IV) with cystic nodal disease (Licitra *et al*., 2006, Ang *et al*., 2010). In view of this, investigation of pre-malignant molecular pathways represents an important research priority, with the ultimate aim to produce a non-invasive screening tool (Psyrri *et al*., 2012) or better evaluation of the risk of pre-invasive disease.

High risk human papillomaviruses (HPV16, 18, 31, 33, 34, 35, 39, 45, 51, 56, 58 and 59) are associated with nearly all cases of uterine cervix carcinoma and ~20% to 30% of HNSCC. As HNSCC also arise in HPV-negative patients (associated with tobacco and alcohol exposure), this type of cancer provides unique opportunities to define similarities and differences based on a viral aetiology. Although HPV16 is thought to be responsible for >95% of viral associated OPSCC, further work is required to investigate the effect of different HPV types on disease outcomes (Chaturvedi *et al*., 2013).

At present, there is a relative paucity of data regarding factors that influence progression of disease from normal epithelium to in situ carcinoma to invasive malignancy. In normal individuals, mucosal HPV carriage in the head and neck varies between 2% - 8% (Duray et al., 2011, Gillison et al., 2012). HPV16 prevalence in the oral cavity is ~1% in the US population (Gillison *et al*., 2012). Age (peak prevalence 30-34 and 60-64 years), sex (males >females), smoking, and multiple sexual partners were independently associated with oral HPV infection in multivariable models. The duration / persistence of oral HPV16 is most likely influenced by local and systemic immune status of an individual (discussed further in chapter 4). Initial studies suggest that most oral HPV infections are likely to be cleared within a year (Chung *et al*., 2014). In OPSCC, Thavaraj *et al*. (2014) indicate that virus-induced field cancerisation and/or multifocal oncogenic HPV infection is uncommon outside of the palatine or lingual tonsil region.

Our methodology reflects previous studies on patients with HPV-associated uterine cervix / anogenital carcinoma. Microarray analysis on fresh frozen tissue samples representing invasive malignancy and normal epithelium may determine novel transcripts - the most clincially relevant of which are then validated using a quantitive analysis e.g. RT-qPCR.

The Illumina platform for whole transcriptome analsyis was chosen primarily due to an existing association with the University of Cambridge (Department of Pathology). Each DNA microarray contains ~50,000 oligonucleotide probes and compares favourably with other market competitors in terms of cost and fidelity of data.

The results described in the chapter have been published in Masterson *et al*. (2015), and some passages have been quoted verbatim from this publication.

3.1.1 Aims of the investigation

This study was designed to identify significant differences in gene expression profiles of HPV-positive (HPV+) and HPV-negative (HPV-) OPSCC and to better understand the functional effects of HPV infection in the pre-malignant pathway.

Twenty-four consecutive patients with locally advanced primary OPSCC were included in a prospective clinical trial. Fresh tissue samples (tumour versus matched normal epithelium) were subjected to whole transcriptome analysis and the results validated on the same cohort with reverse transcription–quantitative real-time PCR (RT-qPCR). In a separate retrospective cohort of twenty-seven OPSCC patients, laser capture microdissection of FFPE tissue allowed RNA extraction from adjacent regions of normal epithelium, carcinoma in situ (pre-malignant) and invasive SCC tissue.

3.2 Materials and Methods

3.2.1 Study population

After informed consent, twenty-four consecutive patients with OPSCC donated multiple fresh biopsy samples (from regions of macroscopically normal and invasive tumour material) at Cambridge University Hospitals NHS Foundation Trust between June 2011 and July 2013 (Table 11). A further twenty-seven OPSCC patients were included from a retrospective cohort and assessed for evidence of carcinoma *in situ* / dysplastic change surrounding invasive carcinoma (Table 12). Disease stage was classified using the AJCC / UICC TNM classification of malignant tumours (Edge *et al*., 2010). Data from this study were deposited in the NIH Gene Expression Omnibus database under accession code GSE56142.

In all prospective fresh biopsy samples, tumour and the adjacent normal tissue were processed to allow DNA and RNA extraction (2.1.2 & 2.1.3). A consultant histopathologist with expertise in head and neck pathology reviewed each specimen to ensure representative sampling (minimum of 75% cancer cells for malignant tissue).

The retrospective FFPE tissue cohort was subjected to laser capture microdissection. This allowed precise RNA extraction from areas of invasive cancer, carcinoma in situ and normal epithelial tissue to facilitate RT-qPCR analysis.

3.2.2 HPV stratification

HPV stratification methods included consensus PGMY PCR (Gravitt *et al*., 2000); type-specific HPV16 DNA PCR and E6/E7 mRNA PCR; DNA sequencing; $p16^{INK4A}$ IHC; and HPV DNA *in situ* hybridisation.

Prospective cohort: Oropharyngeal fresh tissue samples from normal and invasive malignant regions (max. 25 mg) were DNA extracted using a protocol published from

this unit (section 2.1.2) (Winder *et al*., 2009). As previously described (section 2.2.2 & 2.2.3), L1 DNA PCR analysis of tumor DNA (50-100 ng) involved the PGMY09/11 primer set with all negative samples subjected to further amplification using GP5+/GP6+ primers (de Roda Husman *et al*., 1995, Gravitt *et al*., 2000). DNA bands identified after agarose gel electrophoresis were excised, purified using QiaQuick Gel Extraction columns (QIAGEN Ltd, UK) and sequenced directly (section 2.2.4; Source Bioscience, Cambridge, UK). E6/E7 DNA and cDNA PCR analysis involved primers specific for HPV16 E6/E7 (Sotlar *et al*., 2004). For all fresh tissue biopsies, parallel FFPE samples enabled $p16^{INK4a}$ immunohistochemistry (see below).

Retrospective cohort: $p16^{INK4a}$ immunohistochemistry was performed on FFPE tissue using a mouse monoclonal antibody (BD Biosciences, USA) (Masterson *et al*., 2013). DNA *in situ* hybridisation consisted of a probe directed against high risk HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 (Ventana INFORM HPVIII, Tucson, Arizona) (Grogan *et al*., 2006). DNA extraction from FFPE tissue is described in section 2.1.2. L1 and E6/E7 DNA PCR was performed as for fresh tissue samples (above).

3.2.3 RNA Sequencing

The 24 OPSCC patient samples provided multiple fresh biopsy samples at the time of diagnostic or therapeutic surgery. Template RNA samples (up to 1µg in 1µL) were extracted as previously described (section 2.1.3). Whole transcriptome analysis utilized the Illumina GAIIx machine (HumanHT-12 v4 BeadChip) and the results were validated with RT-qPCR (Applied Biosystems ViiA™ 7) (section 2.5.2). All biopsies were selected based on RNA integrity number (RIN) and histopathological review.

3.2.4 Real-Time qPCR

See section 2.5.4 Real-Time qPCR.

3.2.5 Statistical analysis

Statistical calculations were performed using SPSS Version 21 (Chicago, IL, USA). To identify 80% of clinically relevant genes from the Illumina analysis, we based our power calculation on data supplied from Laborde *et al* (Laborde *et al*., 2012). A minimal sample size of 10 subjects in each group were required if FDR (False Discovery Rate) was set at 0.5% and the desired mean log2 fold change >1 (x10 change from baseline) (Li *et al*., 2013). A Pearson's regression coefficient was utilized to investigate any correlation between the Illumina and RT-qPCR analysis (Sabo *et al*., 2008). RT-qPCR data were analyzed by the $2^{-\Delta\Delta CT}$ technique as described previously (Slebos *et al*., 2006). In summary, the average C_t was derived for the three replicate analyses of the reference gene (β-actin), and this was subtracted from the average C_t value from the three replicate analyses for the genes of interest. Expression differences between the HPV+ and HPV- tumors were compared using these normalised $\mathbf{\Delta}C_t$ values and the observed differences subjected to a Student's *t* test. Rates of disease free survival were estimated by means of the Kaplan–Meier method and were compared by the log-rank test. A multivariate model was developed using Cox regression to investigate the effect of clinical factors on disease outcome (HPV16, SYCP2, $p16^{INK4a}$, SFRP1, T stage, N stage, sex, physiological performance status, oropharyngeal subsite, histology grade, smoking, concurrent chemotherapy and age). The main outcome for this study was DFS (disease free survival) which equates to time from completion of treatment until first malignant disease recurrence or death. Overall mortality was also investigated. The whole transcriptome analysis informed which biomarkers may be of relevance. Other secondary outcomes are highlighted in **Table 11 & 12 (p130-133) & Figure 30 (p139)**.

3.3 Results

3.3.1 HPV stratification

In total, 18/24 of the fresh biopsy samples (prospective cohort) and 23/27 of the FFPE samples (retrospective cohort) were classified as HPV positive - defined by evidence of HPV16 L1/E6/E7 DNA, HPV16 E6/E7 mRNA or HPV DNA ISH episomal / integrative staining pattern. Immunohistochemical analysis for expression of $p16^{INK4a}$ was demonstrated for all HPV positive OPSCC samples but also present for 3 out of 10 OPSCC samples categorised as HPV negative (**Table 11 / Table 12**).

3.3.2 DNA & RNA extraction

DNA and RNA extraction from fresh frozen and FFPE specimens is described in section 2.1.2 & 2.1.3. RNA extraction from fresh tissue required x3 biopsy specimens from areas of malignant change versus adjacent normal mucosa. One previous study suggests that stability of HPV16 E6/E7 mRNA improves when the virus is integrated as opposed to episomal (Jeon and Lambert, 1995). The RNA Integrity Number (R.I.N) varied from 1.5 – 8.5 (see section 2.5.1). Of 144 samples collected, 97 displayed an R.I.N of >6 and were deemed suitable for downstream applications (Illumina array / RT-qPCR). RNA extracted from FFPE tissue was of poorer quality in comparison to fresh samples. R.I.N values ranged from 0.4 – 5.2 (R.I.N cut-off value 2) and <40% were deemed suitable for downstream applications (RT-qPCR).

3.3.3 Gene expression differences with HPV status in OPSCC

Among the 47,323 oligonucleotide probes on the DNA microarray, 223 differentially expressed genes were statistically significant in classifying normal versus invasive tissue with further stratification by HPV status (p<0.01; FDR of 0.5%; Figure **28A+B** (p134-35) and Appendix 1). As expected, one of the most significantly expressed

genes in HPV+ tumour tissue was cyclin dependent kinase inhibitor 2A (*CDKN2A;* Log2 fold change 1.7 +/- 0.3 SE), which encodes for $p16^{INK4A}$. This cellular protein may be up-regulated as a result of oncogenic HPV E7 inhibiting activity of retinoblastoma (pRb) (Syrjanen, 2004). Other genes noted to have significant differential expression in the HPV+ group and potential relevance in malignant disease are highlighted in **Table 13** and **Figure 29**.

3.3.4 RT-qPCR

A subset of differentially expressed genes from the Illumina platform analysis was confirmed by RT-qPCR (selected based on clinical relevance in malignant disease). The six target transcripts were: *CDKN2A, SYCP2, SFRP1, DLG2, CRNN and CRCT1*. A high level of agreement existed between the Illumina and RT-qPCR analysis (Pearson correlation coefficient r = 0.905; *p*-value = 0.013; Kolmogorov-Smirnov test of normality $p>0.10$; **Figure 28C**).

In the prospective fresh tissue cohort (Appendix 2), in HPV+ OPSCC, RNA expression levels of *CDKN2A* and *SYCP2* were increased with an average log2 fold change of 1.7 ($p<0.01$; 95% CI 1.0-3.2), and 1.8 ($p<0.01$; 95% CI 1.35-3.35), respectively. Significantly decreased expression was found for *CRCT1* (log2 = -3.0; $p<0.001$; 95% CI 2.2-4.4), *SFRP1* (log2 = -2.3; $p<0.01$; 95% CI 1.7-2.8), *CRNN* (log2 = -2.2; $p<0.01$; 95% CI 1.7-2.9) and *DLG2* (log2 = -1.5; $p<0.01$; 95% CI 1.4-2.2) when compared to normal tissue.

Laser Capture Microdissection (LCM) from the retrospective FFPE cohort allowed analysis of log2 fold change in adjacent regions of normal epithelium, invasive malignancy and carcinoma in situ (**Figure 30A**; Appendix 3 & 4). The results largely reflect the analysis from fresh frozen samples for the HPV+ cohort; *SYCP2* (log2 fold change = 3.1; $p<0.01$; 95% CI, 1.8-4.4) and *SFRP1* (log2 fold change = -0.97; 95% CI, -0.57-1.61) demonstrated the largest differential expression from normal epithelium to pre-malignant (carcinoma in situ) tissue. In the same cohort, 70% of patients displayed significantly elevated expression of *CDKN2A* in the pre-malignant region (log2 fold change >1.5). **Figure 30B** shows comparative data for both fresh

and FFPE tissue designated within the HPV+ category.

3.3.5 Clinical outcomes

The average DFS for all patients was 43.7 months (+/-SE 2.5). For the HPV16+ cohort the DFS period increased to 47.3 months (+/-SE 2.3). Significant expression of *SYCP2* (log2 fold change >1.5) and *SFRP1* (log2 fold change <1.5) cohorts demonstrated an average DFS period of 49.6 months (+/-SE 2.2) and 40.1 months (+/-SE 3.8) respectively. A multivariate proportional hazards model using Cox regression analysis revealed HPV16, *SYCP2*, smoking and physiological performance status to have significant influence on DFS (**Figure 31** & Appendix 5).

To evaluate prognostic accuracy of *SYCP2* expression in regions of HPV+ in situ carcinoma, we constructed a Receiver Operating Characteristic (ROC) curve (Appendix 6). Area Under Curve (AUC) ROC was found to be 0.86 (SE +/- 0.08; 95% confidence interval 0.71–0.99), indicating a good discriminating power when compared to control subjects. Sensitivity and specificity estimates over a range of cut-off points suggest optimal results were obtained for the log2 fold range 1.5-3.0 (Sensitivity 70%; Specificity 95%). Similar testing of *SFRP1* expression revealed the AUC ROC to be 0.64 (SE+/-0.11; 95% confidence interval 0.42–0.86), indicating a poor discriminating power when compared to controls (Appendix 7).

3.4 Discussion

In this prospective observational study, we describe the use of mRNA massive parallel sequencing technology to investigate HPV+/- OPSCC tumors versus matched normal tissue. The data obtained were then validated by RT-qPCR and the results utilized on retrospective FFPE tissue to investigate pre-malignant change surrounding areas of invasive carcinoma.

In all patients, the majority displayed evidence of high risk HPV16 positivity (prospective cohort 75%; retrospective cohort 85%). Predictable fold changes of RNA expression in HPV-associated disease included multiple transcripts within the p53 oncogenic pathway (e.g. *CDKN2A* / *CCND1*). Other candidate transcripts found to have altered levels of expression in this study have not previously been established (*SFRP1, CRCT1, DLG2, SYCP2* and *CRNN*).

SYCP2 showed the most consistent fold change from baseline in pre-malignant tissue and aberrant expression of this protein may contribute to genetic instability during HPV-associated cancer development. If further corroborated, this data may contribute to the development of a non-invasive screening tool.

To date, comparatively few studies have investigated expression profiles in HPV-associated OPSCC (Slebos *et al*., 2006, Martinez *et al*., 2007, Pyeon *et al*., 2007, Walter *et al*., 2013) and even fewer have focused on pre-cancer pathways *(*Mooren *et al*., 2014).

Slebos *et al*. (2006) looked at gene expression profiles of HPV+ and HPV in 36 fresh frozen HNSCC tumour samples (mainly oral cavity). Microarray statistical analysis based on positive HPV16 status (22% of patients) resulted in a list of 91genes that were differentially expressed. Highly expressed genes in HPV16+ samples included those involved with transcription (RFC4, TAF7L, RPA2, and TFDP2), cell cycle regulators (CDC7, p18 and p16INK4A) and meiosis / nuclear structure (SYCP2).

Martinez *et al*. (2007) compared the cellular gene expression profiles of 11 HPV-

positive and HPV-negative oropharyngeal carcinomas with those of the normal oral epithelium (all fresh frozen tissue). Using Affymetrix Human U133A GeneChip, they showed that 397 genes were differentially expressed in HPV-positive HNSCC compared to normal oral epithelium. The up-regulated genes included those involved in cell cycle regulation (CDKN2A), DNA repair (RAD51AP1) and cell differentiation (SFRP4), while down-regulated genes include those involved in proteolysis (PRSS3). 59 differentially expressed genes in HPV-positive HNSCC as compared to both HPV-negative HNSCC and normal oral tissues. Up-regulated genes included those involved in meiosis and nuclear structure (SYCP2), transcription regulation (ZNF238) and DNA repair (RFC5). Genes involved in signal transduction (CRABP2) and proteolysis (KLK8) were found to be down-regulated in HPV-positive HNSCC. GeneChip experiments were validated by quantitative real-time RT-PCR analysis for representative genes.

Pyeon *et al*. (2007) looked at whole genome expression profiles for 42 fresh frozen HNSCC (60% oral cavity, 40% oropharynx), uterine cervix cancers, and site-matched normal epithelial samples. The analysis revealed that HPV positive HNSCC and cervical cancers had different patterns of gene expression yet shared many changes when compared to HPV-negative HNSCC. Some of these were predicted, but many others were not. Notably, HPV-positive cancers over-expressed testis-specific genes that are normally expressed only in meiotic cells (SYCP2 and TCAM1). HPV-positive cancers also demonstrated up-regulation of a larger subset of DNA replication and cell cycle genes than that observed in HPV-negative HNSCC e.g. PC4/SFRS1-interacting protein 1 (PSIP1), V-myb (MYB), synaptogyrin 3 (SYNGR3), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin (SMARCA2) and p16 (CDKN2A). Expression was decreased for genes involved in in epidermal development and hormone activity e.g. cortactin (CTTN), kallikreins (KLK8, KLK10), cyclin D1 (CCND1) and caveolin 1 (CAV1).

Walter *et al*. (2013) used an integrated genomic analysis and validation methodology for 138 HNSCC fresh frozen samples (mainly oral cavity / oropharynx) and identified differential utilization of the lineage markers TP63 and SOX2, deregulation of the NFE2L2 / KEAP1 oxidative stress pathway, and preference for the oncogenes PIK3CA and EGFR. These molecular signatures could have clinical utility by

complementing classification by HPV infection status as well as the high-risk marker CCND1.

Mooren *et al.* (2014) performed p16INK4A immunohistochemistry on FFPE sections for 162 oropharyngeal squamous cell carcinomas (OPSCC), 14 tonsil and 23 laryngeal dysplasias, and 20 tonsil and 27 laryngeal papillomas. Enzyme-immunoassay, FISH and PCR analysis were used to ascertain HPV-presence and type. Of the 14 tonsillar dysplasias and 162 OPSCC, 10 (71%) and 51 (31%) were HPV16+, respectively. p16INK4A immunohistochemistry showed strong nuclear and cytoplasmic staining in 50 out of 51 HPV16+ and 5 out of 111 HPV-negative OPSCC ($p < 0.0001$) and in all HPV16-positive tonsillar dysplasias. In contrast to this, highly variable p16INK4A staining patterns were detected in the papillomas and laryngeal dysplasias, irrespective of HPV-status.

Further characterization of a pre-malignant state in the development of HPV-associated OPSCC would be of clinical value as it infers the potential for a screening test (similar to the uterine cervix carcinoma model). In addition, it may also be of use as a prognostic factor if a biopsy of an abnormal area showed benign hyperplasia or in situ / dysplastic change. In HNSCC surgical excisions, dysplastic epithelium is often found adjacent to the cancer *(*van Zeeburg *et al.*, 2012), but the exact nature of the disease in these regions is poorly defined (Miller and Johnstone, 2001, Jayaprakash *et al.*, 2011).

Within our retrospective Laser Capture Microdissection (LCM) cohort, 70% of patients displayed significantly increased expression of CDKN2A (a proxy marker for HPV infection) in regions of carcinoma in situ relative to normal epithelium. This is higher than the HPV16 estimate (~25% of 458 oral cavity and oropharyngeal dysplasia samples) provided by Jayaprakash *et al* (Jayaprakash *et al.*, 2011) and may be consistent with the hypothesis that HPV plays a significant role in the early phase of oropharyngeal carcinogenesis. Of course, high risk HPV infection can be a transient phenomenon and detection alone may not be sufficient to provide a causal association. Many studies have previously demonstrated the presence of HPV types even in normal oral cavity tissue (Kreimer *et al.*, 2010, Kreimer *et al.*, 2013, Beachler *et al.*, 2015). Gillison *et al.* (2012) performed the largest epidemiological study on this

topic (~6000 subjects), and estimated the prevalence for high-risk HPV types to be 6.9%, of which ~1% can be attributed to HPV16.

It should still be noted that high-risk HPV types (e.g. 16 and 18) in normal oral cavity samples have significantly lower prevalence than the estimates reported by Jayaprakash *et al*. (2011). The available literature also suggests a higher prevalence of HPV16 within areas of *in situ* malignant change when stratified by the male sex (twice that of females) and between areas of transformation from normal through to dysplastic epithelium (three times higher prevalence in dysplastic tissue) (Jayaprakash *et al*., 2011).

In our prospective cohort of 24 OPSCC patients, RNA massive parallel sequencing data provided a statistically significant association for 223 target transcripts stratified by tumor and HPV status. Gene ontology data revealed most transcripts to have limited clinical relevance but a focused analysis of oncological pathways produced several possible transcript candidates e.g. *SYCP2, SFRP1, DLG2, CRNN* and *CRCT1*. Of these, *SYCP2* demonstrated the most significant change from baseline in pre-malignant retrospective FFPE tissue, surpassing the performance of *CDKN2A* (encoding for $p16^{INK2A}$).

The elevated expression of synaptonemal complex protein 2 (*SYCP2*) in HPV-associated tumor tissue has previously been noted in three expression analysis studies (Slebos *et al*., 2006, Martinez *et al*., 2007, Pyeon *et al*., 2007) with elevated log2 fold change values of 2.7, 3.7 and 2.9 respectively. *SYCP2* is a testis-specific human gene and aberrant expression in HPV+ cancers may contribute to the genomic instability induced by high-risk HPVs and subsequent oncogenic change (Pannone *et al*., 2011), alternatively, elevated expression may simply be a result of this instability and de-differentiation of epithelial cells. A hypothetical model applied to HPV+ oropharyngeal carcinoma is provided in **Figure 32** At present, the Wisconsin Alumni Group Foundation has included SYCP2 as one of three target biomarkers in development for HPV associated OPSCC (Lambert *et al*., 2015). To date, no other study has demonstrated elevated expression in pre-malignant tissue. The use of SYCP2 as a prognostic indicator is also of interest given our disease free survival data, however, further research will be required to discern if this is truly independent

of HPV16 expression.

The $p16^{INK4a}$ protein is an inhibitor of cyclin dependent kinase and has increased expression with elevated levels of HPV E7, however, many units have reported a concern regarding false positive results (Schache *et al.*, 2011). Within our HPV16 negative cohort (10/51), three patients had elevated expression of $p16^{INK4a,}$ which may concur with this analysis. At present, stratification of OPSCC tumors by $p16^{INK4a}$ alone is still the preferred approach by most oncology centres as it is cheap, dependable and routinely performed in most analytical laboratories. In an era of OPSCC de-escalation treatments, which are based on a viral etiology, this may incur a risk of undertreating a small proportion of patients falsely considered as HPV-positive (Masterson *et al*., 2014). The use of stepwise algorithms, which combine different HPV assays may compensate for the limitations of individual tests and should now be considered in clinical settings (Robinson *et al*., 2012).

In this study, positive smoking status proved to have significant adverse effect on disease free survival regardless of HPV category (multi-variable analysis $p<0.03$). The negative impact of smoking in HPV+ OPSCC has previously been highlighted by several randomized trials with post-hoc analysis of HPV status (Rischin and Young, 2010, Gillison *et al*., 2012, Posner *et al*., 2011). All studies indicated that the degree of tobacco exposure at diagnosis and during treatment directly correlates with the risk of disease progression and death from malignancy. This may indicate that smoking confers additional tumor mutations in the HPV+ cohort, leading to more aggressive disease and inferior responses to available curative-intent therapies.

However, important questions remain about how to quantify smoking risk to enable comparison between studies. All the major de-escalation trials have largely adopted the arbitrary cut-off point proposed by O'Sullivan *et al.* (O'Sullivan *et al*., 2012) in which 'smokers' are defined as having >10 pack year history. Perhaps more reliable information can be obtained from Laborde *et al.* (2012) who recently published transcription profile data in OPSCC patients stratified by both HPV and smoking status. This indicated that two genes involved in the p53 DNA damage-repair pathway, *ATR* and *CHEK2*, display patterns of increased expression associated with HPV-negative OPSCC smokers only. Our study did not confirm any significant

association with *ATR* or *CHEK2*.

This study is limited by focusing exclusively on whole transcriptome analysis (a single approach), we recognize the need to integrate DNA sequence analysis in future projects (Leemans CR et al., 2011). DNA analysis will demonstrate changes that have occurred to the DNA sequence, whereas mRNA sequence analysis clarifies the effect of those changes. This critically important process therefore identifies which mutations and rearrangements could be the best diagnostic and prognostic indicators. Many current studies display the importance of DNA sequencing in establishing mutations associated with cancer development. The final choice of the six target transcripts in this study may also be open to debate as it is primarily based on gene ontology data supplied through the KEGG (Kanehisa *et al*., 2014) pathway network.

In conclusion, developments in whole-genome sequencing and mRNA analysis are rapidly creating an opportunity to provide personalized information on genetic and functional aspects of malignant tumors. Regarding HPV+ OPSCC, the investigation of differentially expressed genes in normal, pre-malignant and malignant tissue may reveal unique pathways that can explain their different natural history and biological properties. The data from this study reveal *SYCP2* as a potentially important biomarker; if corroborated on a larger scale this may contribute to the development of a non-invasive screening tool e.g. mouthwash or brush biopsy. Current epidemiological data would suggest it is not sufficient to simply screen for OPSCC by presence of HPV16 alone (due to a ~1% carriage rate in the general adult population). Clearly, further well-designed prospective studies are required to confirm this data and to establish if other biomarkers may have future significance.

Table 11 Clinical and histopathological data for the **prospective** cohort (n=24)

HPV-positive status defined as evidence of HPV16 L1/E6/E7 DNA or HPV16 E6/E7 mRNA +/- p16INK4A expression (>70% tumor cell staining); BoT, Base of Tongue; Well, well differentiated SCC; Mod, moderately differentiated SCC; Poor, poorly differentiated SCC; PS, performance status (Eastern Co-operative Oncology Group, ECOG); CRT, chemoradiotherapy; RT, radiotherapy; #, locoregional recurrence; †, non-malignant cause of death; ††, malignant cause of death.

ID	pTNM	Stage	Sex	Subsite	Age	PS	Histol.	Smoker	p16 IHC	DNA seq	PCR HPV16 E6/E7 DNA	PCR (PGMY)	Nested PCR	PCR HPV16 E6/E7 mRNA	Primary treatment	Neck dissection	Clinical f/u (mths)	Clinical outcome
1	T1N2bM0	IVA	F	Tonsil	61	0	Mod	Current	+	16	+	+	+	-	CRT	+	48	-
3	T3N3M0	IVB	M	Tonsil	61	0	Poor	Current	+	16	+	+	+	-	CRT	+	43	-
2	T1N1M0	III	M	BoT	48	0	Poor	Never	+	nil	+	-	-	+	CRT	+	47	-
4	T1N2bM0	IVA	M	BoT	59	0	Well	Current	+	16	-	+	+	-	CRT	+	43	-
5	T3N3M0	IVB	F	BoT	68	3	n/a	Never	+	16	+	+	+	-	n/a	-	14	†
6	T2N1M0	IVA	M	Tonsil	49	1	Poor	Never	+	16	+	-	+	-	RT	-	4	†
7	T1N2bM0	IVA	M	BoT	65	0	Poor	Never	+	nil	+	+	+	+	CRT	+	43	-
8	T2N2bM0	IVA	M	BoT	52	0	Poor	Never	+	16	+	-	+	+	CRT	+	43	-
9	T2N2cM0	IVA	M	BoT	57	1	n/a	Never	+	16	+	+	+	-	CRT	-	40	-
10	T1N2bM0	IVA	M	BoT	75	1	Mod	Former	+	16	+	+	+	+	RT	+	39	-
11	T2N1M0	IVA	M	Tonsil	47	0	Poor	Never	+	16	+	+	+	-	CRT	+	39	-
12	T1N1M0	III	F	Tonsil	60	0	Mod	Former	+	16	+	+	+	+	RT	-	39	-
13	T3N2aM0	IVA	M	Tonsil	56	2	Poor	Never	+	33	+	+	+	+	CRT	+	37	-
14	T1N2bM0	IVA	M	BoT	78	1	Poor	Former	+	16	+	-	+	-	RT	+	37	-
15	T1N2aM0	IVA	M	Tonsil	59	0	n/a	Former	+	16	-	+	+	-	RT	+	36	-
16	T1N2aM0	IVA	M	Tonsil	55	0	Mod	Current	+	nil	+	+	+	+	CRT	+	35	-
17	T2N2bM0	IVA	M	Tonsil	58	0	Well	Current	+	16	+	+	+	+	CRT	+	30	-
18	T1N2bM0	IVA	M	BoT	81	1	Poor	Former	+	16	+	+	+	+	RT	-	12	††
19	T2N2bM0	IVA	M	Tonsil	53	0	Poor	Never	+	n/a	-	-	-	-	CRT	-	40	-
20	T1N1M0	IVA	M	Tonsil	42	0	n/a	Former	+	n/a	-	-	-	-	CRT	-	41	-
21	T3N0M0	III	M	BoT	56	1	Mod	Current	-	n/a	-	-	-	-	CRT	-	15	# 15 mths
22	T3N1M0	III	M	Tonsil	43	1	Mod	Current	-	n/a	-	-	-	-	CRT	-	13	††
23	T1N2bM0	IVA	M	Tonsil	77	1	n/a	Former	-	n/a	-	-	-	-	RT	+	37	-
24	T1N3M0	IVB	M	BoT	77	1	mod	Former	-	n/a	-	-	-	-	RT	+	38	# 22 mths

HPV positive prospective cohort
HPV negative prospective cohort

Table 12: Clinical and histopathological data for the **retrospective** cohort (n=27)

HPV-positive status defined as evidence of HPV16 L1/E6/E7 DNA or episomal / integrative pattern on HPV DNA ISH +/- p16 expression. Concordant regions of normal, in situ and invasive carcinoma were subjected to further analysis to discern mRNA expression of the six target biomarkers (CDKN2A, SYCP2, SFRP1, DLG2, CRNN and CRCT1). BoT, Base of Tongue; Well, well differentiated SCC; Mod, moderately differentiated SCC; Poor, poorly differentiated SCC; PS, performance status (Eastern Co-operative Oncology Group, ECOG); CRT, chemoradiotherapy; RT, radiotherapy; #, locoregional recurrence; †, non-malignant cause of death; ††, malignant cause of death.

ID	pTNM	Stage	Sex	Subsite	Age	PS	Histo.	Smoker	p16 IHC (1st)	p16 IHC (2nd)	DNA seq	PCR HPV16 E6/E7	PGMY PCR	Nested PCR	DNA ISH (1st)	DNA ISH (2nd)	Reviewers concord. for invasive carcinoma	Reviewers concordant for in situ carcinoma	Primary Rx	Neck dissection	Clinical f/u months	Clinical outcome
1	T1N0M0	I	M	Tonsil	67	0	?	Former	+	+	16	-	-	+	-	-	-	+	RT	-	51	-
2	T2N2bM0	IVA	M	BoT	57	0	Poor	Current	+	+	16	+	+	+	+	+	+	+	RT	-	44	-
3	T2N2aM0	IVA	M	Tonsil	52	0	Poor	Current	+	+	16	+	+	+	+	+	+	+	CRT	+	44	-
4	T1N2bM0	IVA	F	Tonsil	63	1	Poor	Never	+	+	16	+	+	+	+	+	+	+	CRT	+	44	-
5	T1N1M0	III	F	Tonsil	67	1	Poor	Never	+	+	16	-	+	+	+	+	+	+	RT	+	40	-
6	T1N2aM0	IVA	M	BoT	47	0	Poor	Never	+	+	16	+	+	+	+	?	+	+	CRT	+	48	-
7	T1N2bM0	IVA	F	BoT	49	0	Poor	Never	+	+	16	+	+	+	+	+	+	+	CRT	+	51	-
8	T1N1M0	III	F	Tonsil	40	0	Poor	Current	+	+	33	+	+	+	+	+	+	+	CRT	-	40	-
9	T1N0M0	I	M	BoT	77	1	?	Former	+	+	16	+	+	+	-	+	-	+	RT	-	7	†
10	T1N2aM0	IVA	F	Tonsil	62	0	Poor	Never	+	+	16	+	+	+	+	?	+	+	RT	+	42	-
11	T3N2bM0	IVA	M	Tonsil	72	1	Mod	Former	+	+	16	-	+	+	+	+	-	-	RT	+	11	††
12	T2N2bM0	IVA	M	BoT	64	0	Mod	Former	+	+	16	+	+	+	+	+	+	-	CRT	+	47	-
13	T2N1M0	III	M	BoT	51	0	Mod	Never	+	+	16	+	+	+	+	+	+	-	CRT	-	53	-
14	T3N2cM0	IVA	M	BoT	33	0	Mod	Never	+	+	18	+	+	+	+	?	-	-	CRT	-	4	-
15	T1N2bM0	IVA	M	Tonsil	61	0	Poor	Current	+	-	16	-	-	+	+	+	+	-	RT	+	41	-
16	T2N2aM0	IVA	M	Tonsil	58	0	Mod	Never	+	+	16	+	+	+	+	-	+	-	CRT	+	39	-
17	T1N2aM0	IVA	M	Tonsil	46	0	Poor	Current	+	+	16	-	+	+	+	?	-	-	RT	+	38	-
18	T1N1M0	III	M	BoT	53	0	?	Never	-	-	16	+	+	+	-	?	-	-	CRT	-	37	-
19	T2N2cM0	IVA	F	BoT	51	0	Poor	?	+	+	16	+	+	+	+	?	+	-	CRT	+	44	-
20	T2N1M0	III	M	Tonsil	53	1	?	Former	+	+	16	+	+	+	-	?	+	-	CRT	+	41	# 10 mths
21	T3N2cM0	IVA	M	Tonsil	51	0	Poor	Current	+	+	16	+	+	+	+	?	-	-	CRT	-	12	††
22	T4aN0M0	IVA	M	Tonsil	82	1	Poor	Never	+	+	16	+	+	+	-	+	+	-	RT	-	4	†
23	T2N2cM0	IVA	M	BoT	40	0	Mod	Never	+	+	16	-	-	+	+	+	+	-	CRT	-	13	††
24	T2N2aM0	IVA	F	Tonsil	56	0	Mod	Current	-	-	nil	-	-	-	-	-	+	+	CRT	+	48	-
25	T1N2aM0	IVA	M	Soft pal.	61	2	Poor	Current	-	-	nil	-	-	-	-	-	+	+	RT	+	34	# 16 mths
26	T3N1M0	III	M	BoT	71	0	Mod	?	-	-	?	?	?	?	-	-	+	-	CRT	-	35	# 11 mths
27	T1N1M0	III	M	BoT	60	0	Poor	?	-	+	?	?	?	?	-	?	+	-	CRT	+	37	-

HPV positive retrospective cohort
HPV negative retrospective cohort

Figure 28A Two-way analysis of variance identified target sequences with significant differential expression between normal and tumor tissue and further stratified by HPV status. The volcano plot x-axis is the $\log_2$ fold-change value (tumor versus normal epithelium) and the y-axis is –log10 odds value (HPV-positive versus negative cohort). The horizontal line represents a log odds value, $p<0.01$ (FDR of 0.5%), as the threshold cutoff. Samples highlighted in red have significantly increased expression within tumour tissue compared to normal controls. Samples highlighted in green have significant negative expression.

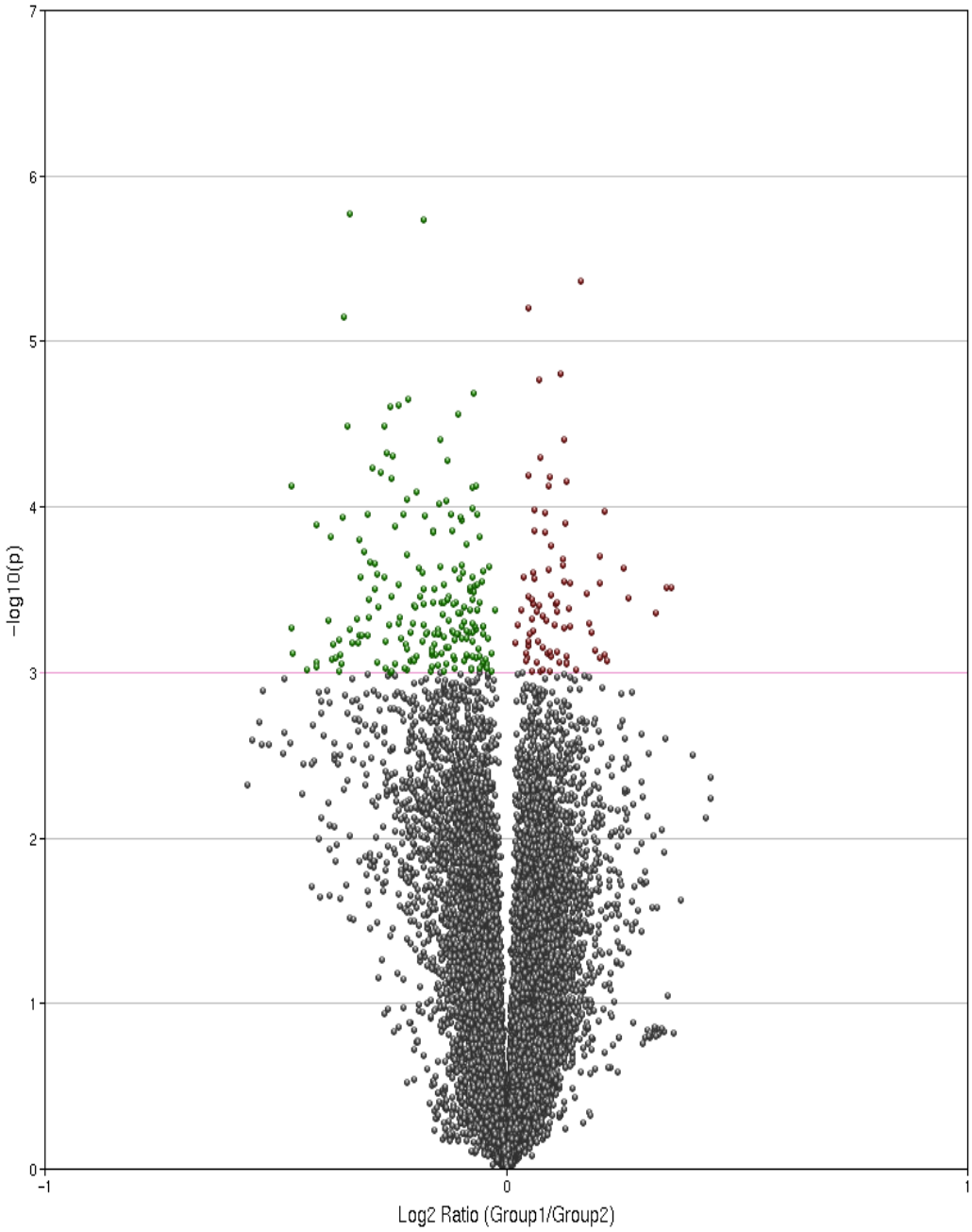


Figure 28B Hierarchical clustered heatmap of 223 genes displaying significant differential expression. As expected, *CDKN2A*, ranked highly on full transcriptome analysis. Five other genes that may have clinical relevance in HPV+ malignant disease are *SYCP2*, *SFRP1*, *CRCT1*, *CRNN* and *DLG2*.

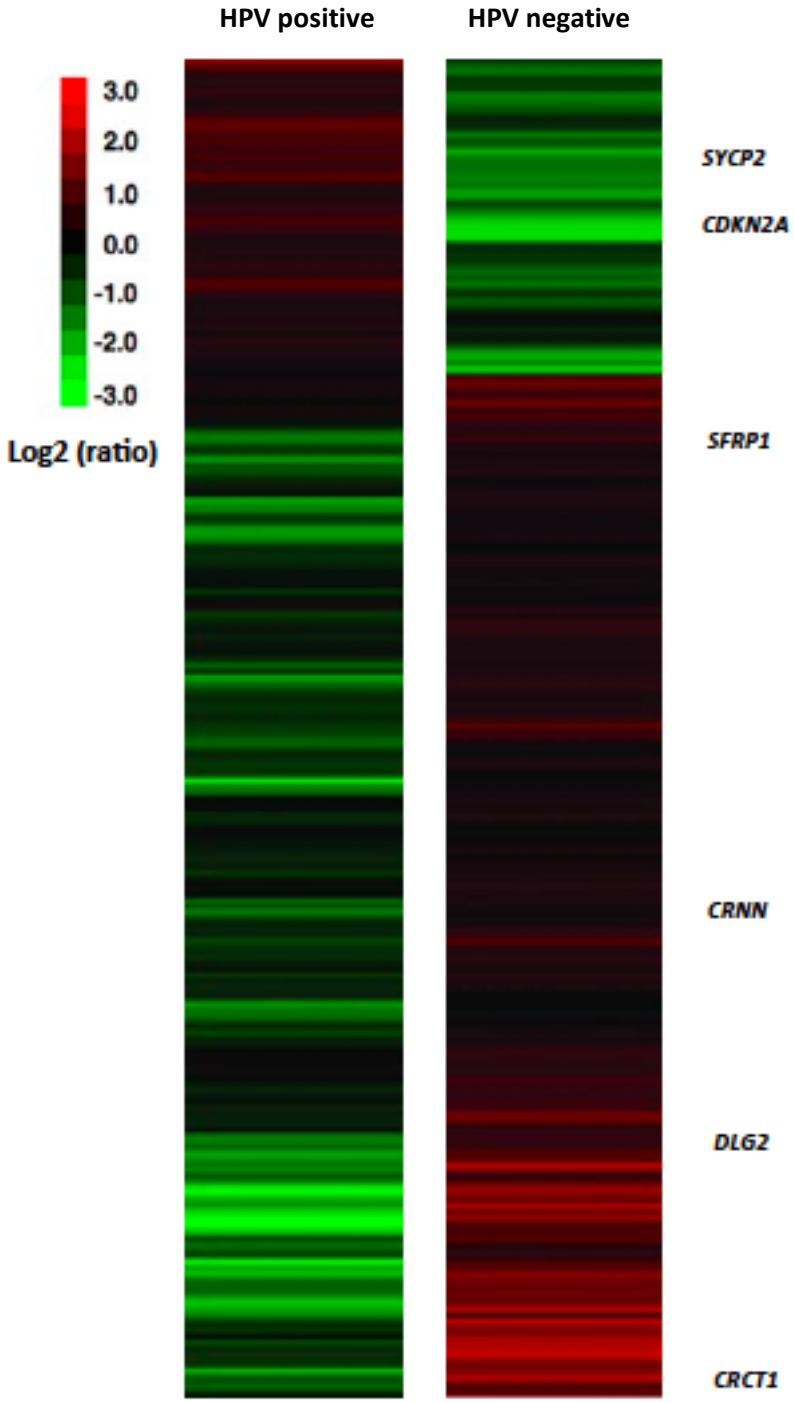


Figure 28C Validation graph for expression analysis (Illumina versus RT-qPCR; Pearson correlation coefficient r = 0.905; *p*-value = 0.013; Kolmogorov-Smirnov test of normality *p*>0.10) (Masterson *et al*., 2015). Standard Error Mean (SEM) bars calculated by Log2 fold change difference between normal and invasive carcinoma tissue: - sqrt(se_A²+se_B²).

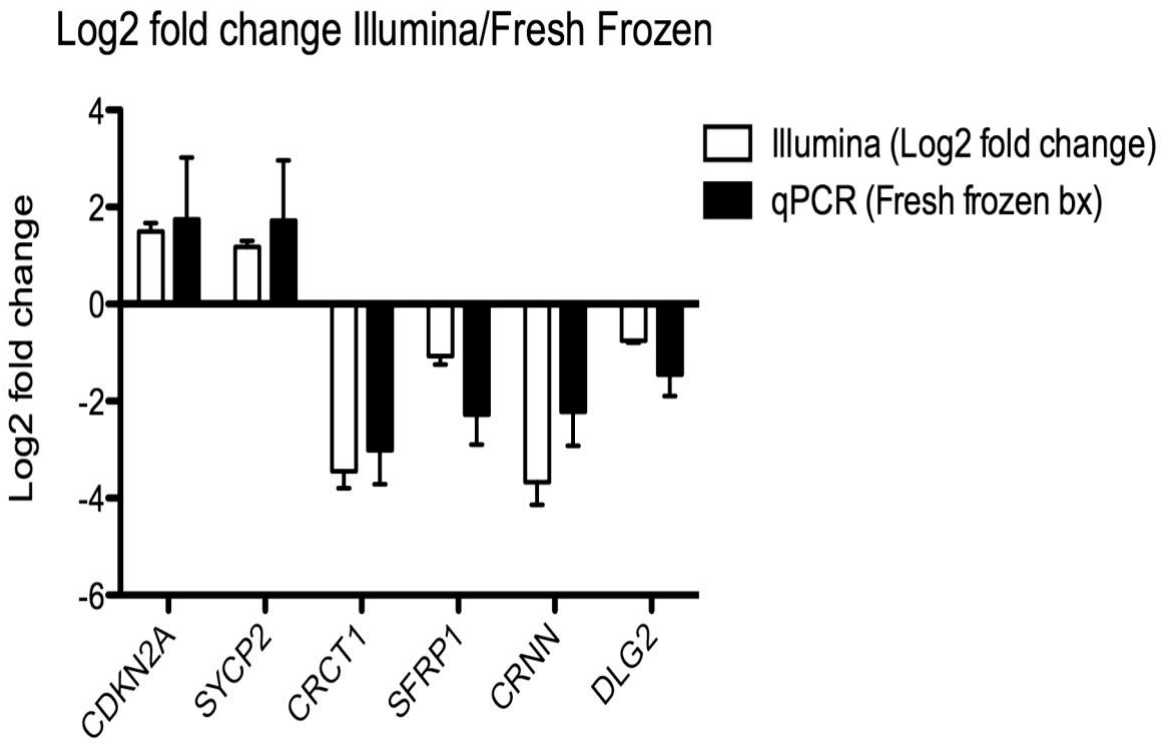


Table 13: Genes of Interest

Gene symbol (expression value)	**Rank** (1-223)	**Description (novel transcript in HPV+ OPSCC)**
CRNN (-3.6 +/- SE 0.5)	**169**	CRNN [cornulin) is a recently identified gene encoding a protein with an S100 EF-hand calcium-binding motif, and its expression is known to be down-regulated in esophageal squamous cell carcinoma. CRNN overexpression in oral squamous carcinoma negatively regulates cell proliferation by the induction of G1 arrest.
CRCT1 (-3.5 +/- SE 0.3)	**223**	CRCT1 (cysteine-rich C-terminal 1) is a protein-coding gene, and has been validated by a similar gene expression profiling study for cervical carcinoma. Down regulation of CRCT1 [also known as NICE1] may predispose to oncogenic change.
SFRP1 (-1.2 +/- SE 0.2)	**35**	This gene encodes a member of the Secreted Frizzled Related Protein (SFRP) family that contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzled proteins. Members of this family act as soluble modulators of Wnt signaling; epigenetic silencing of SFRP genes leads to deregulated activation of the Wnt-pathway, which is associated with cancer. The role of SFRP1 as a tumour suppressor has been proposed in many cancers based on its loss in patient tumors.
DLG2 (-0.7 +/- SE 0.1)	**218**	This PDZ domain gene has not been investigated previously but DLG1 and DLG4 have demonstrated a significant interaction with HPV16 E6 in cervical carcinoma. This gene encodes a member of the membrane-associated guanylate kinase (MAGUK) family. The encoded protein forms a heterodimer with a related family member that may interact at postsynaptic sites to form a multimeric scaffold for the clustering of receptors, ion channels, and associated signaling proteins.
SYCP2 (1.5 +/- SE 0.2)	**16**	SYCP2 (synaptonemal complex protein 2) is a protein-coding gene. SYCP2, an SYCP1 homologue, is a component of the meiotic synaptonemal complex like structure. Increased expression of these meisosis specific proteins in testicular cancer may contribute to the genomic instability and subsequent oncogenic change.
Gene symbol (expression value)	**Rank** (1-223)	**Description (established transcript in HPV+ OPSCC)**
CDKN2A (1.7 +/- SE 0.3)	**32**	Cyclin-dependent kinase inhibitor 2A (CDKN2A) is also known as $P16^{INK4A}$. It is a tumor suppressor protein that is encoded by the CDKN2A gene. CDKN2A / $P16^{INK4A}$ plays an important role in regulating the cell cycle and is significantly over-expressed in response to HPV E7.
CCND1 (-1.2 +/- SE 0.1)	**136**	The CCND1 gene encodes for the protein Cyclin D1. Cyclins function as regulators of CDKs (Cyclin-dependent kinases). This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Cyclin D1 has been shown to interact with Retinoblastoma (Rb), which is a tumor suppressor protein. Cyclin D1 expression should be down regulated in HPV-positive head and neck squamous cell carcinoma because of pRb suppression.

Figure 29A The initial analysis focused on the P53 oncogenic pathway in HPV positive disease, where we found predictable and significant fold changes for both p16 and Cyclin D1 (highlighted in red font).

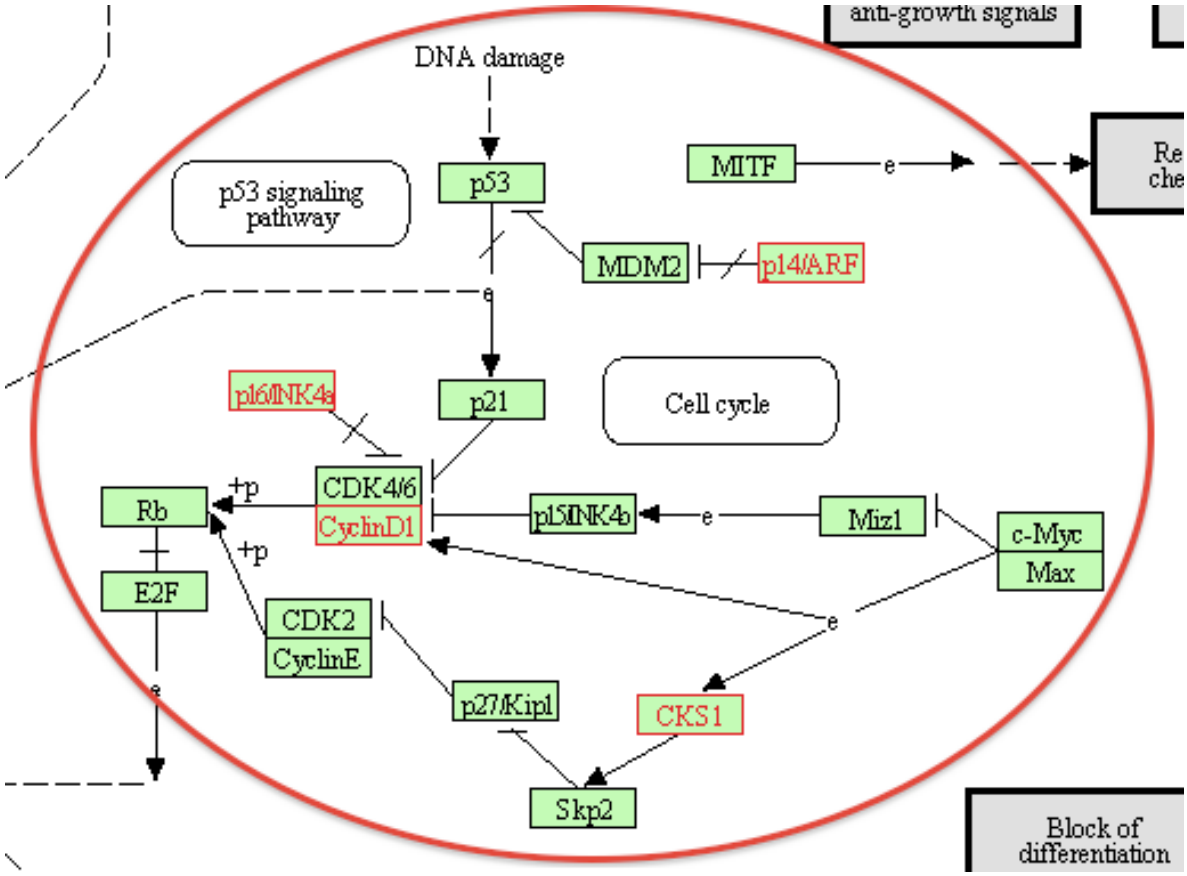


Figure 29B Our search then expanded to explore other known cancer pathways which may or may not contribute to this condition. All the gene transcripts highlighted in red font are represented on the Illumina array and differentially expressed in our samples according to invasive versus normal tissue with further stratification by HPV status. P53 oncogenic pathway highlighted by red circle.

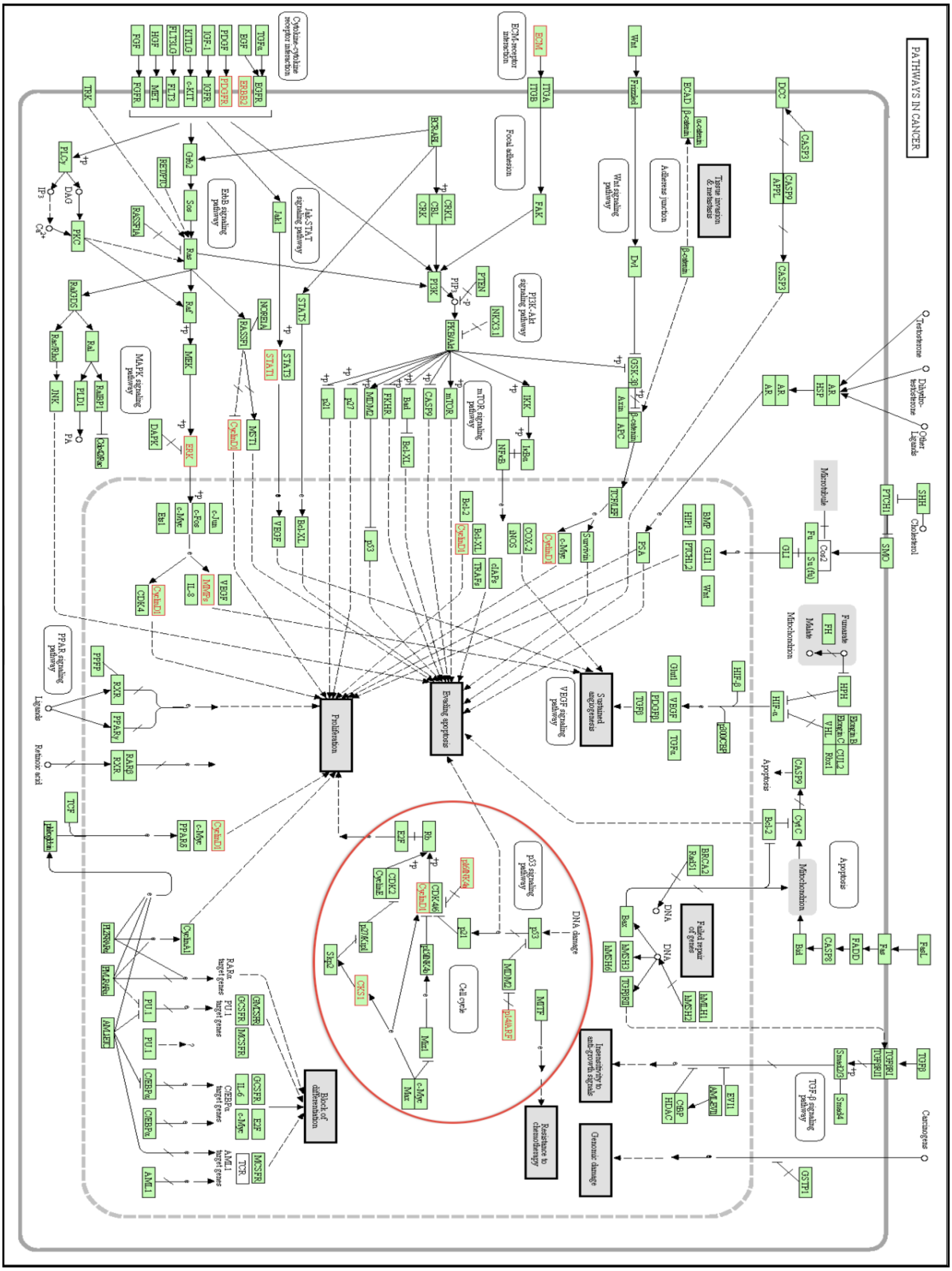

PATHWAYS IN CANCER
Proliferation
Evading apoptosis
Sustained angiogenesis
Tissue invasion & metastasis
Block of differentiation
Resistance to chemotherapy
Genomic damage
Insensitivity to anti-growth signals
Failed repair of genes

Figure 30A H&E staining showing the junction between normal epithelium and severe dysplasia in HPV+ tonsil SCC (top left) (Masterson *et al.*, 2015). MCM7 (top right) and Ki67 (bottom left) positive stains are present in >2/3rd of the abnormal epithelium but restricted to the basal layers in normal epithelium. In situ hybridisation reveals an elevated level of HPV 16 DNA (bottom right) in the dysplastic epithelium when compared to the normal region (x100 Olympus BX51 [inset x150], Infinity capture software).

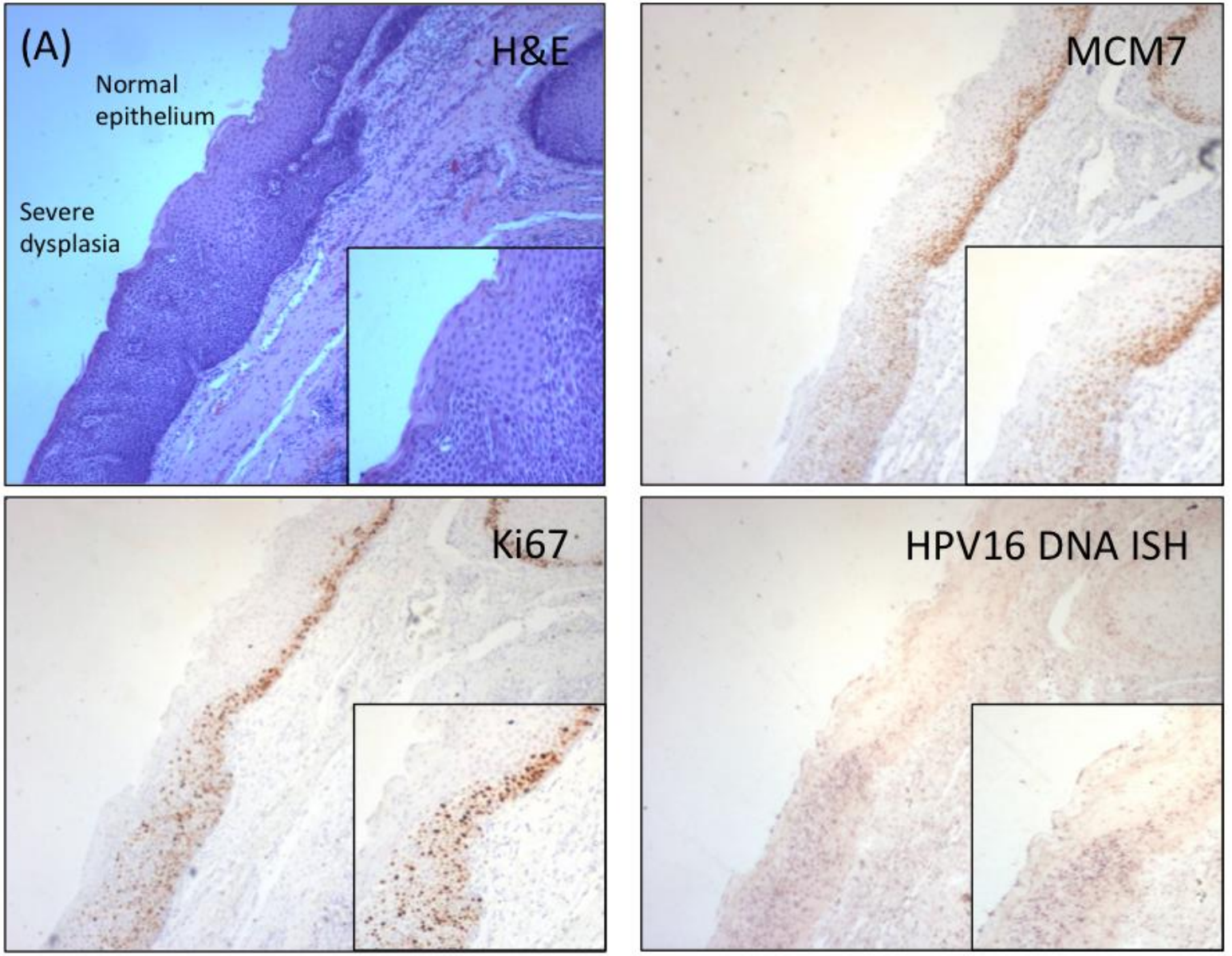

(A)
Normal epithelium
Severe dysplasia
H&E
MCM7
Ki67
HPV16 DNA ISH

Figure 30B RT-qPCR analysis for all HPV+ sample types showing significant differential expression of SYCP2 (elevated log2 fold change = 3.1 [95% CI 1.8-4.4]; $p<0.01$) in pre-malignant (in situ) compared to normal tissue (Masterson *et al*., 2015). Standard Error Mean (SEM) bars calculated by Log2 fold change difference between normal and invasive carcinoma tissue:- sqrt(se_A²+se_B²).

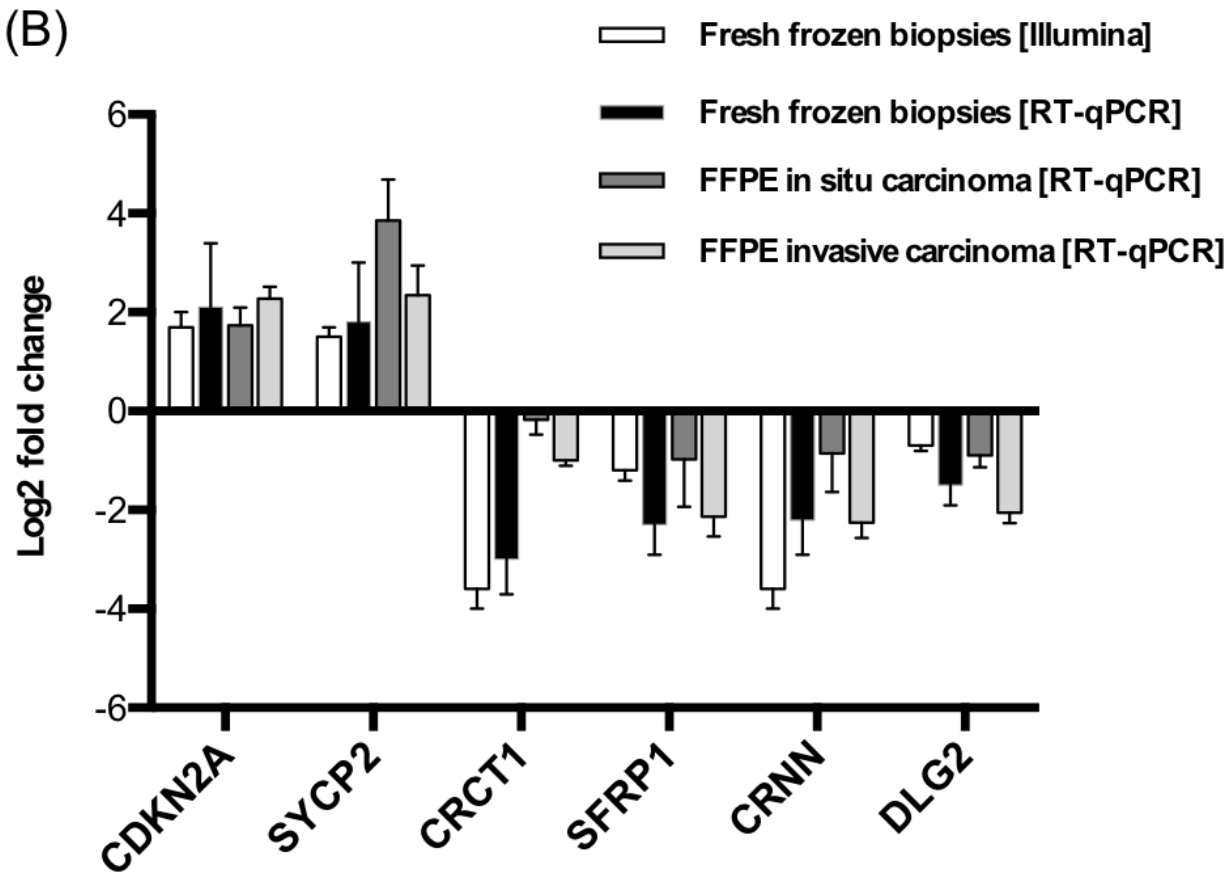
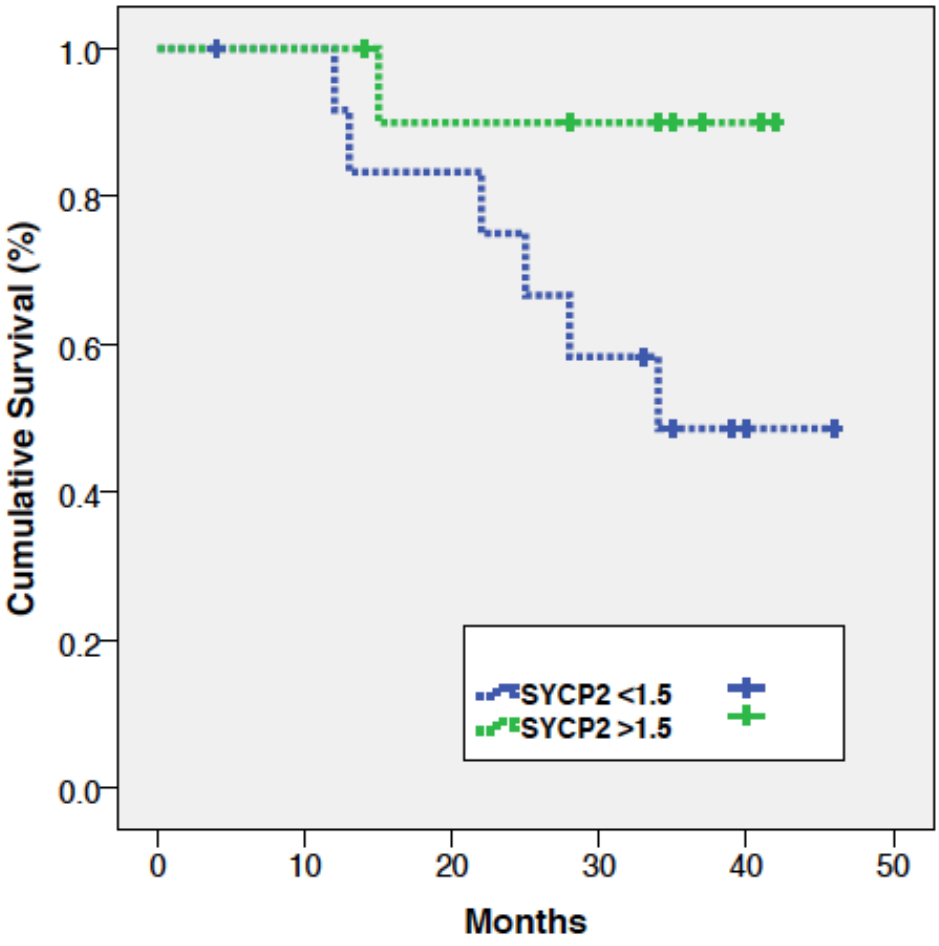


Figure 31 Disease free survival calculations from Kaplan-Meier survival analysis (SYCP2 $p<0.02$ / HPV16 $p<0.02$). Cox regression to investigate the effect of clinical multiple parameters on disease free survival retained HPV16 and SYCP2 in the final model (see Figure S5) (Masterson *et al.*, 2015).



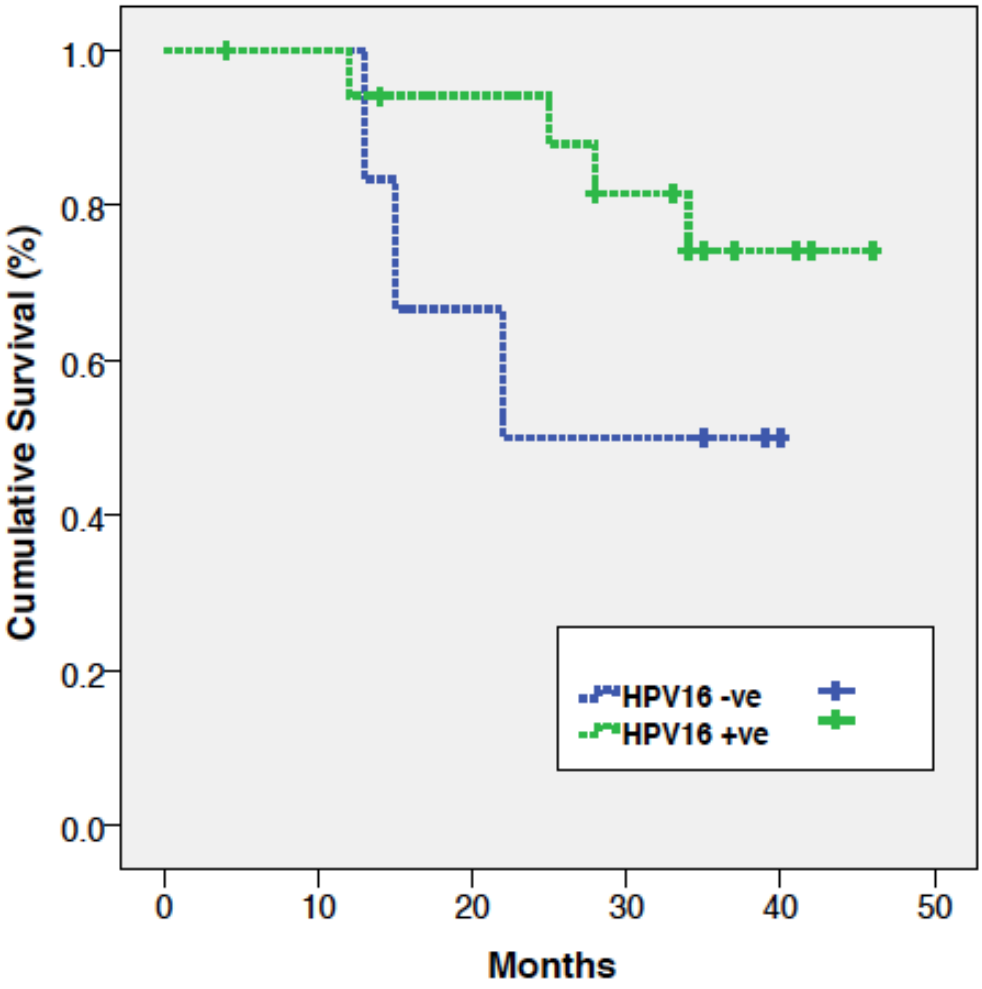
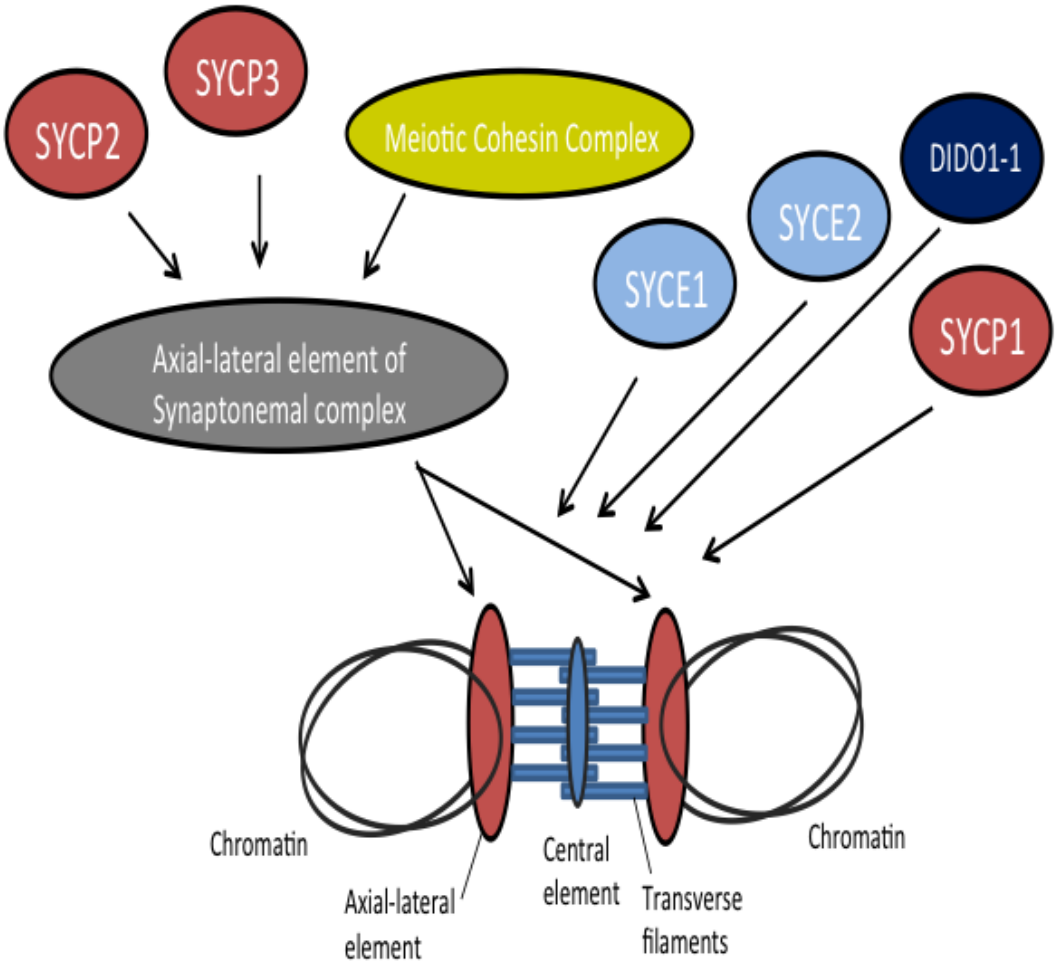

Cumulative Survival (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Months
HPV16 -ve
HPV16 +ve

Figure 32 Hypothetical model for SYCP2 (synaptonemal complex protein 2) regarding HPV+ OPSCC. SYCP2 is a protein that is involved in the linkage of chromosomes via the synaptonemal complex, binding DNA at the scaffold attachment regions and driving the prophase of meiosis. Alterations in the gene expression of SYCP2 have been associated with impaired meiosis (Masterson *et al.*, 2015).



Chapter 4

Investigation of CD4+ & CD8+ cell response to HPV E2, E6 and E7 in patients with OPSCC

4.1 Introduction

Infection with human papillomavirus can be of variable duration and may or may not be associated with overt clinical symptoms. Cutaneous HPV lesions generally manifest as warts and will often resolve over a period of months to years. Anogenital infections may remain asymptomatic or hidden for some time e.g. CIN and AIN pre-malignant stages.

DNA amplification-based tests demonstrate that anogenital infections are quite frequent and often self-limiting (Garland *et al*., 2016). Impaired cell mediated immunity may result in failure to clear or control infection, and in the case of the high risk (oncogenic) HPV, an increased likelihood of developing invasive carcinoma. Viral evasion of innate immunity, which in turn delays activation of the adaptive immunity, seems to be correlated to duration of HPV associated disease (Stanley, 2012a).

Animal models show that specific antibodies formed against the virus coat protein L1 are protective, which suggests that this may be an effective prophylactic vaccine strategy (Frazer, 2009). Virus like particle (VLP) vaccines circumvent epithelial immune evasion strategies by undergoing intramuscular delivery to the body. VLPs are reported to generate significant levels of B cell memory, as indicated by persistence of the antibody and robust recall responses (Stanley, 2008).

HPV16 and 18 are responsible for ~70% of uterine cervix cancers, although multiple other oncogenic types increase the HPV positivity rate to ~99% (Garland *et al*., 2016). High risk HPV infections are now also accepted as major aetiological agents in squamous cell carcinoma of the anus, vulva, vagina, penis and more recently the oropharynx. HPV is considered to be a causal agent in ~5% of human cancers with HPV16 by far the most common type (de Martel *et al*., 2012).

The results described in the chapter have been published in Masterson *et al*. (2016a), and some passages have been quoted verbatim from this publication.

4.1.1 Immune response to HPV associated anogenital disease

Regarding anogenital HPV disease, significant effort has been directed towards understanding the natural history of the host's immune response. Evidence gained from the study of immune compromised patients (e.g. HIV and renal transplant recipients) demonstrate the importance of cell-mediated immunity (CMI) in control of HPV infection; often documenting a higher prevalence of disease in this cohort. Such individuals show increased frequency of anogenital HPV infection and increased HPV persistence in the body (Palefsky, 2008). Infection with multiple oncogenic HPV types are also more common (Hernandez *et al*., 2014).

Investigation of CMI responses to HPV has been hampered by multiple factors. First, HPV infection is specifically located to squamous epithelial sites, with little systemic manifestation. Second, gene transcription patterns of the HPV life cycle are intricately related to the stages of differentiation of keratinocytes. Only in recent years have successful models allowed *in vitro* propagation of HPV (Stanley, 2012a).

Innate immunity

Epithelial cells, once viewed principally as a physical barrier, are now regarded as having much more complex immunological roles. Cervical keratinocytes secrete low levels of a variety of pro-inflammatory cytokines, chemokines and growth factors, and can be induced by various stimuli (Van den Bergh and Guerti, 2014).

Innate immunity in the anogenital model is now understood to heavily involve tumour necrosis factor (TNF), transforming growth factor β (TGF-β), and the type I interferons (IFN-α and IFN-β), which are produced by epithelial and other cell types (Stanley, 2012a, Songock et al., 2017).

Investigation of the role of Natural Killer (NK) cells in the aetiology of HPV associated anogenital disease suggests a protective function. $HPV16^+$ infected keratinocytes in subjects with carcinomas have been correlated to decreased NK cell lysis (Brestovac *et al.*, 2013, Malejczyk *et al.*, 1993). NK cells can destroy virally infected tumour cells via killer immunoglobulin-like receptors (KIR) that recognise ameliorated MHC class I expression. Decreased sensitivity of PBMCs to immunostimulatory cytokines such as IL-2 and IFN-α (as seen in chronic infection or increased PD1 expression) may lead to a reduced immune response to $HPV16^+$ infected keratinocyte and thus poorer clinical outcomes (Papasavvas *et al.*, 2016).

Adaptive immunity

Adaptive cell mediated immunity involves the recognition and effector phases of epithelial immune responses demonstrated in both cutaneous and mucosal HPV-infected tissues (Frazer, 2009). These include clonally expanded T cells that have returned to infected epithelial tissues via mechanisms involving adhesion molecules and chemokines; Langerhans cells, which capture antigens for transfer to regional lymph nodes and presentation to naive T cells / macrophages.

Recognition phase

Depleted immune surveillance may be due to reduction in Langerhans cells because of normal egress or other unidentified mechanisms. Prolonged infection and oncogenic change has been linked to the depletion of Langerhans cells in HPV infected epithelial cells, along with other local immune deficiencies (Doorbar *et al.*, 2015).

Once Langerhans cells have captured antigen, the recognition phase continues with their transfer to locoregional lymph nodes. Cytokines which are principally derived from HPV infected keratinocytes are important mediators of this process. These include TNF and IL-1α (primarily from keratinocytes) and IL-1β (primarily from Langerhans cells). IL-10, which

acts as an inhibitor of Langerhans cell migration, is produced by keratinocytes. The initiation of Langerhans cell maturation into dendritic cells is promoted by granulocyte-macrophage colony-stimulating factor, also produced by keratinocytes (Moerman-Herzog *et al*., 2015).

Effector phase

Proliferation and related studies of T-cell responsiveness to HPV

T-cell proliferative responses and IL-2 release help to determine the role of helper T lymphocytes (CD4+) in providing protection against the development of HPV-associated lesions (Doorbar *et al*., 2015, Stanley, 2012a).

Studies to date have analysed helper T-lymphocyte responses to HPV-16 antigens, as HPV-16 is the commonest oncogenic HPV type (Bosch *et al*., 2006). Due to the roles of the E6 and E7 in cell transformation, immune responses to HPV16 E6 and E7 proteins and peptides have been widely studied (Stanley, 2012a). Subjects with normal cytology have been proven to display a more frequent response to these antigens in comparison to those who have developed pre-malignant or invasive uterine cervix SCC (Steele *et al*., 2005).

A study by Woo *et al*. (2010) investigated T-cell response to the gene products of HPV-16 E2, E6 or E7 using a longitudinal design. This demonstrated that subjects were more likely to have cleared their HPV infection and less likely to develop pre-malignant change if positive responses to either E2 or E6 peptides were demonstrated. de Jong and van Poelgeest (2004) also observed that baseline HPV16 specific $CD4^+$ helper T-cell response is either absent or severely impaired in uterine cervix cancer patients (despite a relatively robust immune status).

Studies of CTL-mediated killing

Major histocompatibility complex (MHC) class I-restricted CTLs which are CD8+, are known to be responsible for the recognition and killing of virus-infected host cells and virus-induced tumours (Frazer, 2009).

Analysis of uterine cervix pre-malignant and malignant specimens has displayed the presence of activated CTL in lesions using immunohistochemical staining (Origoni *et al.*, 2013). Sirianni et al. (2004) revealed that $CD8^+$ T cells reactive to wild-type p53 (more usually associated with HPV+ malignant disease) are significantly reduced after surgical excision of HPV16 tumours.

HPV immune evasion strategies

Observational studies demonstrate that HPV can effectively evade recognition by the immune system for many months (Pett *et al.*, 2006). The HPV infectious cycle itself may be responsible for this as very low levels of viral protein are expressed - resulting in no or limited viraemia. Virus replication and assembly only occur in cells already destined for "programmed death", with limited resultant inflammation and no excitatory signal to engage the immune system (Stanley, 2012a).

High risk HPV E6 and E7 proteins actively suppress the activation of interferon response genes; interferon being a key antiviral defence mechanism (Kanodia *et al.*, 2007). Further to this, the E7 proteins have been demonstrated to downregulate Toll Like Receptor 9 which effectively evades the innate immune response delaying the activation of adaptive immunity (Hasan *et al.*, 2007).

4.1.2 Immune response to HPV-associated oropharyngeal carcinoma

In head and neck squamous cell cancer, immunodeficiency has been shown to correlate with a poor prognosis (Ferris, 2015). However, few studies have addressed the specific role of cell mediated immunity in HPV-associated OPSCC (Wansom *et al*., 2010, Ramos *et al*., 2013). Within this context, specific questions to be addressed include susceptibility / persistence of HPV infection and progression to malignancy.

Spanos *et al*. (2009) showed that a functional immune response was critical to achieving adequate tumour clearance with chemoradiation in a murine model. Hoffmann *et al*. (2010) investigated $CD8^+$ T cells reactive to HPV16 E7 in a small cohort of patients with OPSCC, this specific T-cell response (HPV16 E7) was commonly detected in subjects with a positive HPV16 status but the study lacked survival analysis as a clinical outcome measure.

Most of the studies above focus on immune response at the point of diagnosis and prior to curative therapy. At present, it is unclear whether conventional treatment protocols for OPSCC (Mirghani *et al.*, 2015) inhibit or promote tumour immune response mechanisms (Freiser *et al*., 2013). Distel and Buttner (2012) investigated intra-tumoral immune profiles before and after primary chemoradiation and concluded that post-therapy cytotoxic T lymphocytes (CTL) were depleted to a lesser extent than immunosuppressive T regulatory cells. This contrasts with the findings of Al-Taei *et al*. (2013) who found decreased systemic HPV specific T cell responses and accumulation of immunosuppressive influences in oropharyngeal cancer patients following radical therapy

Similar to anogenital disease, natural regulatory T cells (T_{reg}) are important immunosuppressive cells and have been correlated with a poor prognosis in HNSCC (Karimi *et al*., 2015). They have an important role in homeostasis and can be characterised by high co-expression of $CD4^+$ and $CD25^+$(Smigiel *et al*., 2014). T_{reg} inhibit T cell activity by induction of ATP hydrolysis and apoptosis via the Fas / FasL pathway (Motz *et al*., 2014). An increasing body of evidence would suggest they are responsible for ameliorating tumour-specific immune responses (Nishikawa *et al*., 2005), and as such are a potential barrier to

immunotherapy (Hoffmann *et al.*, 2010, Lindau *et al.*, 2013). Although some immunotherapies will specifically target T regulatory cells e.g. IL2.

4.1.3 Aims of the investigation

Immunological response to human papillomavirus in the development and progression of HPV16+ OPSCC (accounting for most viral associated cases) is largely unknown and may provide important insights for new therapeutic strategies e.g. adjuvant immunotherapeutic approaches that target viral antigens E6/E7 may act systemically to reduce microscopic foci of malignant disease.

In this prospective clinical trial (UKCRN11945), specific cell-mediated immune responses to HPV16 E2, E6 and E7 in peripheral blood using IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT) were examined before and after radical treatment. $CD56^+$, $CD4^+$, $CD8^+$ and regulatory T cell frequencies were also discerned by flow cytometry.

4.2 Materials and Methods

4.2.1 Study population

The study comprised fifty-one patients with primary oropharyngeal squamous cell carcinoma and eleven control subjects with benign disease. All patients were treated with curative intent by radiotherapy +/- chemotherapy. Disease specific survival was investigated by multivariate analysis. Disease stage was classified using the AJCC / UICC TNM classification of malignant tumours (Edge *et al*., 2010).

A baseline sample of the tumour was taken for histological analysis and the remainder processed for DNA +/- RNA extraction. A consultant histopathologist with expertise in head and neck pathology reviewed each tissue block to ensure adequate tumour sampling. Clinical data for all subjects are shown in **Table 14A+B**. The outcomes observed for this study were DFS (Disease Free Survival) stratified by CD4+ / CD8+ response to HPV16 E2, E6 & E7 epitope (see section 2.1.2). The average follow up length was 43.7 months.

4.2.2 Lymphocyte cell preparation

In each patient, a blood sample (40ml) was drawn at baseline after diagnosis and 3 months post treatment. To examine the specificity of the IFN-γ ELISPOT, healthy patients were recruited to the study, each of whom underwent tonsillectomy for a benign condition.

4.2.3 Blood sample collection for ELISPOT assay.

See section 2.6.1

4.2.4 ELISPOT assays for IFN-γ

See section 2.6.2

4.2.5 Flow cytometry

See section 2.6.3 (Fluorescent activated cell sorting (FACS) analysis).

4.2.6 HPV stratification

See section 2.2 (Detection of HPV in clinical samples).

4.2.7 Statistical analysis

Statistical calculations were performed using SPSS Version 21 (Chicago, IL, USA). Rates of disease free survival were estimated by means of the Kaplan–Meier method and were compared by the log-rank test. A two-tailed p value of <0.05 was defined as significant.

4.3 Results

4.3.1. HPV-specific T cell responses correlate with tumour HPV status

In total, 41/51 of tumour samples were classified as HPV positive - defined by evidence of HPV16 L1/E6/E7 DNA or HPV16 E6/E7 mRNA +/- HPV DNA ISH episomal / integrative staining pattern. Immunohistochemical analysis for expression of $p16^{INK4a}$ was demonstrated for all HPV+ OPSCC samples but also present for 2 out of the 10 OPSCC samples categorised as HPV negative (**Table 14A+B)**.

Data summarising HPV status and T cell response are shown in Appendix 8 & 9. When linking HPV16 status with the ELISPOT data, we found that 80% (33/41) of patients who had detectable HPV16 DNA in tumours also had $CD4^+$ or $CD8^+$ T cell response to HPV16 E6/E7. Only one patient with a HPV negative tumour displayed such a response, suggesting a correlation is likely between HPV positive status of the tumour and T cell response to HPV antigens.

4.3.2 $CD4^+$ and $CD8^+$ T cell response to HPV16 E7 reduce post-treatment for the majority of patients

Specific IFN-γ responses to HPV16 E2, E6 & E7 by both $CD4^+/CD8^+$ T-cells were investigated at initial diagnosis of malignancy and three months after completion of chemoradiation for 39/51 patients. Pre-treatment $CD4^+$ response to E2 was detected for eight patients (8/30); this frequency decreased slightly after treatment (8/39). One patient demonstrated a $CD8^+$ response to E2 prior to treatment only.

Overall, $CD8^+$ T cell responses to HPV16 E6 and E7 peptides were detected pre-treatment in 60% (18/30) and 70% (21/30) of patients, respectively. Post-treatment evaluation revealed there was no significant change in $CD8^+$ response to E6 (39.5%; 15/38; $p<0.16$) and a significant decreased response to E7 peptide (44.7%; 17/38; $p<0.02$; **Figure 32**).

Pre-treatment $CD4^+$ T cell responses to HPV16 E6 and E7 peptides were detected in 60% (18/30) and 70% (21/30) of patients, respectively. Post-treatment, there was a significant decrease in ELISPOT responses to both E6 (35.9%; 14/39; $p<0.02$) and E7 peptides (43.6%; 17/39; $p<0.01$; **Figure 33**).

To ascertain sensitivity and specificity of the IFN-γ assay, patients undergoing tonsillectomy for benign disease were also subjected to the same analysis at baseline. All displayed no positive IFN-γ response for either $CD4^+$ or $CD8^+$ T-cells except for one subject (1/11). The same subject displayed evidence for HPV16 L1 DNA on initial HPV stratification.

IFN-γ production by CD56 cells (NK cell population) was utilised to discern non-specific (innate) immune response within the clinical subgroups. Appendix 10 shows no significant difference was detected for all patients pre- or post-treatment.

4.3.3 Decreased CD4:CD8 ratio following radical therapy

The CD4:CD8 ratio pre-treatment was similar to that seen in healthy controls (3.03 vs. 2.87). However, $CD4^+$ (0.74 vs. $0.42x10^9$/L) and $CD8^+$ (0.37 vs. 0.33 $x10^9$/L) cell numbers were reduced post-therapy with a larger proportionate decrease in $CD4^+$ T cells ($p<0.04$; **Figure 33**). Haemoglobin level (13.1 vs. 12.1 g/dl), total lymphocytes (1.4 vs. 1.1 $x10^9$/L), monocytes (1.6 vs. 1.3 $x10^9$ /L) and platelet count (298 vs. 307 $x10^9$ /L), all remained stable before and after treatment.

4.3.4 $CD4^+CD25^+CD127^{low/-}$ T_{reg} cell frequencies increase in HPV+ OPSCC patients after treatment

Frequency of $CD4^+$ cells with high co-expression of $CD25^+$ and low co-expression of CD127 were examined for most patients (34/51) using gate-settings as previously described (**Figure 35** & Appendix 11) (Baecher-Allan *et al.*, 2001, Ormandy *et al.*, 2005). Although the

proportion of $CD4^{+}CD25^{+}CD127^{low/-}$ T_{reg} cells did not differ at baseline between the patient groups, there was significant elevation in HPV+ OPSCC patients after treatment (pre-treatment 6.6% +/-SE 0.9 versus post-treatment 9.5% +/-SE 0.6 $p<0.03$).

4.3.5 Improved disease free survival with elevated CD8+ T cell response to HPV16 E7

The average DFS for all patients was 43.7 months (+/-SE 2.5). For the HPV16+ cohort, the DFS period increased to 47.3 months (+/-SE 2.3). In those with an increased $CD8^{+}$ T cell response to E7, the average DFS period was 49.6 months (+/- SE 2.3; **Figure 36** & Appendix 12). A multivariate proportional hazards model using Cox regression analysis revealed HPV16 ($P<0.02$), retained or enhanced $CD8^{+}$ T cell response to E7 ($p<0.03$) and smoking ($p<0.04$) to have significant influence on DFS.

4.4 Discussion

Oropharyngeal squamous cell carcinoma is the possible outcome of infection with high-risk human papillomavirus (HPV) and is preceded by a phase of persistent HPV infection during which the host immune system fails to eliminate the virus. Fortunately, most oral cavity HPV infections are cleared before oncogenic conversion (Gillison *et al*., 2012, Garland *et al*., 2016).

In this prospective observational study, we report a systematic analysis of IFN-γ response in primary oropharyngeal carcinoma. The significant finding of this dataset would suggest an attenuated response by $CD4^+$/$CD8^+$ T-cells to HPV16 peptides after completion of radical therapy. Specific factors found to influence disease free survival include HPV16 status, smoking and post-treatment $CD8^+$ response to HPV16 E7.

As yet, most HNSCC studies investigating interaction between T-cell response and the HPV16 E6 or E7 oncoproteins are small and confined to a non-clinical setting (Sirianni *et al*., 2004, Hoffman *et al*., 2006, Spanos *et al*., 2009, Wansom *et al*., 2010, Al-Taei *et al*., 2013, Ramos *et al*., 2013, Ferris, 2015). This contrasts with the more extensive research evaluating specific immune response in uterine cervix cancer (Andersson *et al*., 2000, de Jong and van Poelgeest, 2004, Kaufmann *et al*., 2007, Roman *et al*., 2007, Farhat *et al*., 2009, Stanley, 2012a).

The improved clinical outcome with retained $CD8^+$ response to HPV16 E7 may contradict data from previous studies that show a more prominent immunogenic role for E6 (Farhat *et al*., 2009, Nakagawa *et al*., 2000). This situation may have arisen due to the more preserved status of E7 (compared to E6) in pre-malignant and malignant disease (Andersson *et al*., 2000). E7 has also been used as the target epitope for many therapeutic vaccination trials for CIN (Roman *et al*., 2007).

HPV E6 is transcribed from the same transcript as E7 and thus may be be expressed in

similar frequencies and quantity. It may be reasonable to assume a similar antiviral T-cell response, particularly for E6*1, which is the splice isoform of E6 expressed at high levels (along with E7) in tumours. E7 is expressed at the expense of full length E6, therefore a spectrum of responses to E7 and E6*1 and not full length E6 in tumours is possible (Tang *et al*., 2006).

$CD8^+$ cytokine response to the HPV16 E7 epitope was more likely in our HPV16 RNA+/DNA+ group - as such it may simply act as a proxy marker for improved clinical outcome regardless of the immune phenotype. However, this specific response could have clinical relevance as CD8+ T-cells are the dominant immune killer cells for virus-infected cells, intracellular bacteria and cancer cells. Previous studies (Albers *et al*., 2005, Hoffman *et al*., 2006) also indicate that patients with HPV16+ HNSCC exhibit an increased number of T cells specific for peptides derived from the HPV E7 oncoprotein. Also, in contradiction to our study findings, HPV-specific T-cell responses (as a marker of HPV infection) would presumably lead to *generalised* correlations with improved survival for **most or all** CD4+ and CD8+ T-cell responses (E2, E6 & E7).

The reduced T cell responses after treatment noted in this study cannot be explained by a concurrent decrease in lymphocytes or memory T cells; absolute numbers for both groups remained unaffected after radical therapy (**Figure 34**). Instead, there was a significant increase in regulatory T cells (T_{reg}) noted within the HPV16+ cohort before and after treatment (11.6% versus 15.4%; $p<0.02$). These data suggest that immunosuppression (due to increased regulatory T cell activity) can influence HPV-specific immune response post-treatment; in addition, they would support murine models where an immunodeficient host failed to clear HPV16 positive tumour cells after exposure to cisplatin (Spanos *et al*., 2009).

Schuler *et al*. (2013) have demonstrated a higher T_{reg} frequency and a lower CD8+/T_{reg} ratio post CRT with no stratification by HPV status. The study authors suggest that the T_{reg} fraction in PBMC is relatively resistant to CRT, unlike the CD4+ and CD8+ T-cells. This hypothesis was tested in vitro and demonstrated supportive data. Our study indicates that increased frequency of T_{regs} (post-treatment) is restricted to HPV+ cohort (Figure 35). If

confirmed to be the case, important drivers of this association may include pathways related to PDZ-domain proteins (E6), pRB, p107, p130, wildtype p53 or E7.

Although disease free survival was not significantly influenced by T_{reg} frequency before and after treatment, further functional studies may be beneficial. In particular, investigation of PD1 or PDL-1 expression from TILs (Tumour infiltrating lymphocytes) pre- or post-treatment may be of clinical value (Lyford-Pike *et al.*, 2013). A previous study has demonstrated increased PD1 expression from TILs is linked to improved survival in HPV+ OPSCC (Badoual *et al.*, 2013). It may be of interest to determine whether changes in (or selection for) a PD1-high tumour cell population drives the balance between cytotoxic and regulatory T cells (**Figure 37**).

Our data reveal two patients assigned to the HPV- OPSCC cohort tested positive for $p16^{INK4A}$. Potential reasons for this observation include intra-observer / inter-observer variation of the immunohistochemical background stain. In addition, a small but significant cohort of the false positive HPV- samples have $p16^{INK4A}$ mutations that may account for accumulation of inactive $p16^{INK4A}$ (Romagosa and Simonettis, 2011). These data reinforce previous evidence that p16+ status alone is not a safe or justifiable method of assigning HPV status to a tumour (Robinson *et al.*, 2012, Masterson *et al.*, 2014, Mirghani *et al.*, 2015).

Limitations of this research are the small sample size and the possible insensitivity of the assay due to the localised nature of the infection. The reduced cohort size may result in less power to detect associations with E6 or E7. Problems with detection of Cell Mediated Immunity (CMI) response in peripheral blood are not surprising since HPV16 is a specific oropharyngeal subsite infection and consequently the number of circulating memory cells would be hypothesized to be small (in addition a decreased systemic immune response may be secondary to viral suppression). The E2 antigen may also have a more important role in viral control at an earlier stage of infection (Davidson *et al.*, 2003). It would be of interest to obtain information regarding sustainability of the IFN-γ ELISPOT response at a more distant time-point after primary treatment (>5-10 years). We postulate that this may continue to decrease as antigen exposure becomes diminished (with clearance of infection or the cancer).

Conversely, a rising $CD4^+$ / $CD8^+$ T cell response may indicate recurrence of disease.

In summary, we have found that the frequency of IFN-γ secreting $CD4^+$ / $CD8^+$ T-cells specific for HPV16 E6 and E7 peptides were decreased in most patients 3 months after radical therapy. Further to this, we observe a significantly improved disease free survival outcome in the small subset of patients who retain or enhance their $CD8^+$ E7 response. HPV16 and smoking status were also found to correlate with survival. Increased immunosuppressive influences after treatment (for the HPV16 cohort) are supported by a concomitant rise in T_{reg} frequencies. Although the E6 and E7 epitopes are apparently immunogenic in patients with HPV-associated oropharyngeal carcinoma, it is unclear why specific T-cells are unable to eliminate or prevent oncogenic change at an earlier stage. Further studies are required to explain this resistance of tumour targets to cytotoxic T cells and to find potential strategies that will increase the chances of developing HPV-based therapeutic vaccine in the future (**Figure 38**).

Table 14A: Clinical and histopathological data (HPV positive)

OPSCC patients had a mean age of 58 years and the male-female ratio was 4:1. The majority had local advanced disease (stage IVA ~70%). No patient had distant metastases (stage IVC). Treatment involved chemotherapy (cisplatin 100 mg/m^2, days 1, 22, 43) given concurrently with radiation therapy (65 Gy in 35 fractions to both primary and nodal sites). In two subjects with significant renal or cardiac disease, cetuximab 400 mg/m^2 IV loading dose 1 week before the start of radiation therapy, then 250 mg/m^2 weekly. For patients with limited nodal disease (usually >3cm), a selective or modified radical neck dissection was performed before the start of chemoradiation. The multi-disciplinary team assessed clinical response based primarily on surface measurements at direct laryngoscopy, supplemented by radiographic imaging (^{18}F-fluoro-2-deoxyglucose positron emission tomography / computed tomography [FDG PET/CT]). Disease free survival and overall survival were determined after a median period of 43 months. BoT, Base of Tongue; Well, well differentiated SCC; Mod, moderately differentiated SCC; Poor, poorly differentiated SCC; PS, Eastern Co-Operative Group (ECOG) physiological performance status; CRT, chemoradiotherapy; RT, radiotherapy. Clinical outcome: #, locoregional recurrence; †, non-malignant cause of death; ††, malignant cause of death; n/a, not available.

Study ID	pTNM	Stage	Sex	Subsite	Age	PS	Histol.	Smoker	p16 IHC	DNA seq	PCR HPV16 E6/E7 DNA	PCR (PGMY)	Nested PCR	PCR HPV16 E6/E7 mRNA	DNA ISH	Primary treatment	Neck dissection	Clinical f/u (mths)	Clinical outcome
1	T3N3M0	IVB	M	Tonsil	61	0	Poor	Current	+	16	+	+	+	-		CRT	+	43	
2	T1N1M0	III	M	BoT	48	0	Poor	Never	+	nil	+	-	-	+		CRT	+	47	
3	T3N2bM0	IVA	M	Tonsil	72	1	Mod	Former	+	16	-	+	+		+	RT	-	11	††#
4	T1N2bM0	IVA	M	BoT	59	0	Well	Current	+	16	-	+	+	-		CRT	+	43	
5	T3N3M0	IVB	F	BoT	68	3	n/a	Never	+	16	+	+	+	-		RT	-	14	†
6	T1N2bM0	IVA	F	BoT	49	0	Poor	Never	+	16	+	+	+		+	CRT	+	51	
7	T3N2cM0	IVA	M	BoT	33	0	Mod	Never	+	18	+	+	+		+	CRT	-	4	
8	T1N1M0	III	F	Tonsil	67	1	Poor	Never	+	16	-	+	+		+	RT	+	40	
9	T2N2bM0	IVA	M	BoT	57	0	Poor	Current	+	16	+	+	+		+	RT	-	44	
10	T1N2bM0	IVA	M	BoT	65	0	Poor	Never	+	nil	+	+	+	+		CRT	+	43	
11	T1N2bM0	IVA	F	Tonsil	61	0	Mod	Current	+	16	+	+	+	-		CRT	+	48	
12	T1N0M0	I	M	BoT	77	1	n/a	Former	+	16	+	+	+		-	RT	-	7	†
13	T3N2cM0	IVA	M	Tonsil	51	0	Poor	Current	+	16	+	+	+		+	CRT	-	12	††#
14	T2N1M0	IVA	M	Tonsil	49	1	Poor	Never	+	16	+	-	+	-		RT	-	4	†
15	T2N2cM0	IVA	M	BoT	57	1	n/a	Never	+	16	+	+	+	-		CRT	-	40	
16	T2N2bM0	IVA	M	BoT	64	0	Mod	Former	+	16	+	+	+		+	CRT	+	47	
17	T1N2bM0	IVA	M	BoT	75	1	Mod	Former	+	16	+	+	+	+		RT	+	39	
18	T2N1M0	IVA	M	Tonsil	47	0	Poor	Never	+	16	+	+	+	-		CRT	+	39	
19	T1N1M0	III	F	Tonsil	60	0	Mod	Former	+	16	+	+	+	+		RT	-	39	
20	T1N2aM0	IVA	F	Tonsil	62	0	Poor	Never	+	16	+	+	+		+	RT	+	42	
21	T4aN0M0	IVA	M	Tonsil	82	1	Poor	Never	+	16	+	+	+		-	RT	-	4	†
22	T2N2bM0	IVA	M	BoT	52	0	Poor	Never	+	16	+	-	+	+		CRT	+	43	
23	T2N2bM0	IVA	M	Tonsil	58	0	Well	Current	+	16	+	+	+	+		CRT	+	30	
24	T2N1M0	III	M	BoT	51	0	Mod	Never	+	16	+	+	+		+	CRT	-	53	
25	T2N2cM0	IVA	M	BoT	40	0	Mod	Never	+	16	-	-	+		+	CRT	-	13	††#
26	T3N2aM0	IVA	M	Tonsil	56	2	Poor	Never	+	33	+	+	+	+		CRT	+	37	
27	T1N2aM0	IVA	M	Tonsil	46	0	Poor	Current	+	16	-	+	+		+	RT	+	38	
28	T1N1M0	III	M	BoT	53	0	n/a	Never	-	16	+	+	+		-	CRT	-	37	
29	T1N2bM0	IVA	M	BoT	78	1	Poor	Former	+	16	+	-	+	-		RT	+	37	
30	T2N2aM0	IVA	M	Tonsil	58	0	Mod	Never	+	16	+	+	+		+	CRT	+	39	
31	T1N2aM0	IVA	M	Tonsil	55	0	Mod	Current	+	nil	+	+	+	+		CRT	+	35	
32	T1N2bM0	IVA	M	BoT	81	1	Poor	Former	+	16	+	+	+	+		RT	-	12	††#
33	T1N2aM0	IVA	M	Tonsil	59	0	n/a	Former	+	16	-	+	+	-		RT	+	36	
34	T1N2bM0	IVA	M	Tonsil	61	0	Poor	Current	+	16	-	-	+		+	RT	+	41	
35	T2N2cM0	IVA	F	BoT	51	0	Poor	n/a	+	16	+	+	+		+	CRT	+	44	
36	T2N1M0	III	M	Tonsil	53	1	n/a	Former	+	16	+	+	+		-	CRT	-	41	# 10 mths
37	T1N1M0	III	F	Tonsil	40	0	Poor	Current	+	33	+	+	+		+	RT	-	40	
38	T1N0M0	I	M	Tonsil	67	0	n/a	Former	+	16	-	-	+		-	RT	-	51	
39	T2N2aM0	IVA	M	Tonsil	52	0	Poor	Current	+	16	+	+	+		+	CRT	+	44	
40	T1N2bM0	IVA	F	Tonsil	63	1	Poor	Never	+	16	+	+	+		+	CRT	+	44	
41	T1N2aM0	IVA	M	BoT	47	0	Poor	Never	+	16	+	+	+		+	CRT	+	48	

Table 14B: Clinical and histopathological data (HPV negative)

Study ID	pTNM	Stage	Sex	Subsite	Age	PS	Histol.	Smoker	p16 IHC	DNA seq	PCR HPV16 E6/E7 DNA	PCR (PGMY)	Nested PCR	PCR HPV16 E6/E7 mRNA	DNA ISH	Primary treatment	Neck dissection	Clinical f/u (mths)	Clinical outcome
42	T1N2aM0	IVA	M	Soft pal.	61	2	Poor	Current	-	nil	-	-	-		-	RT	+	34	# 16 mths
43	T2N2aM0	IVA	F	Tonsil	56	0	Mod	Current	-	nil	-	-	-		-	CRT	+	48	
44	T1N1M0	III	M	BoT	60	0	Poor	n/a	-	n/a	n/a	n/a	n/a		-	CRT	+	37	
45	T3N1M0	III	M	BoT	71	0	Mod	Current	-	n/a	n/a	n/a	n/a		-	CRT	-	35	# 11 mths
46	T3N0M0	III	M	BoT	56	1	Mod	Current	-	n/a	-	-	-	-		CRT	-	15	#15mths
47	T2N2bM0	IVA	M	Tonsil	53	0	Poor	Never	+	n/a	-	-	-	-		CRT	-	40	
48	T1N1M0	IVA	M	Tonsil	42	0	n/a	Former	+	n/a	-	-	-	-		CRT	-	41	
49	T3N1M0	III	M	Tonsil	43	1	Mod	Current	-	n/a	-	-	-	-		CRT	-	13	††
50	T1N2bM0	IVA	M	Tonsil	77	1	n/a	Former	-	n/a	-	-	-	-		RT	+	37	
51	T1N3M0	IVB	M	BoT	77	1	mod	Former	-	n/a	-	-	-	-		RT	+	38	#22mths

Figure 33: HPV16 E2, E6 & E7 response detection by IFN-γ ELISPOT pre and post radical therapy (Masterson *et al*., 2016a).

A response was considered positive if the average number of spot forming cells (SFC) in HPV antigen well was 2 standard deviations (SD) above the average of negative control. The frequency of cytokine secreting $CD4^+$ / $CD8^+$ / $CD56^+$ cells were then derived by the formula: number of spots/number of cells per well. For this study, we assume that increased staining of $CD4^+$ equates to $CD4^+$ T cells; we assume that increased staining of $CD8^+$ equates to $CD8^+$ T cells.

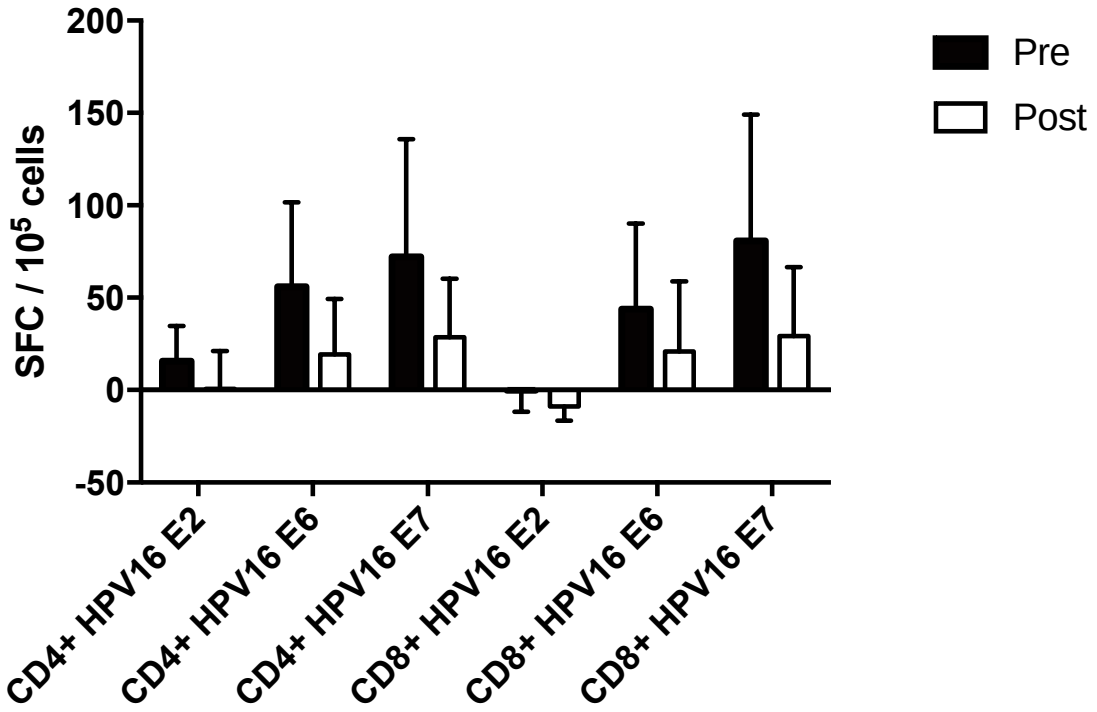


Figure 34: Mean haematological parameters (+/- SD) in OPSCC patients (n=51) before and after radical therapy (Masterson *et al.*, 2016a). The graph shows changes in A) CD4 & CD8 % B) haemoglobin C) lymphocytes D) monocytes and E) platelets. The CD4:CD8 ratios were calculated using the frequencies of CD4+ and CD8+ population. All other subgroups were taken from the routine FBC in the clinic.

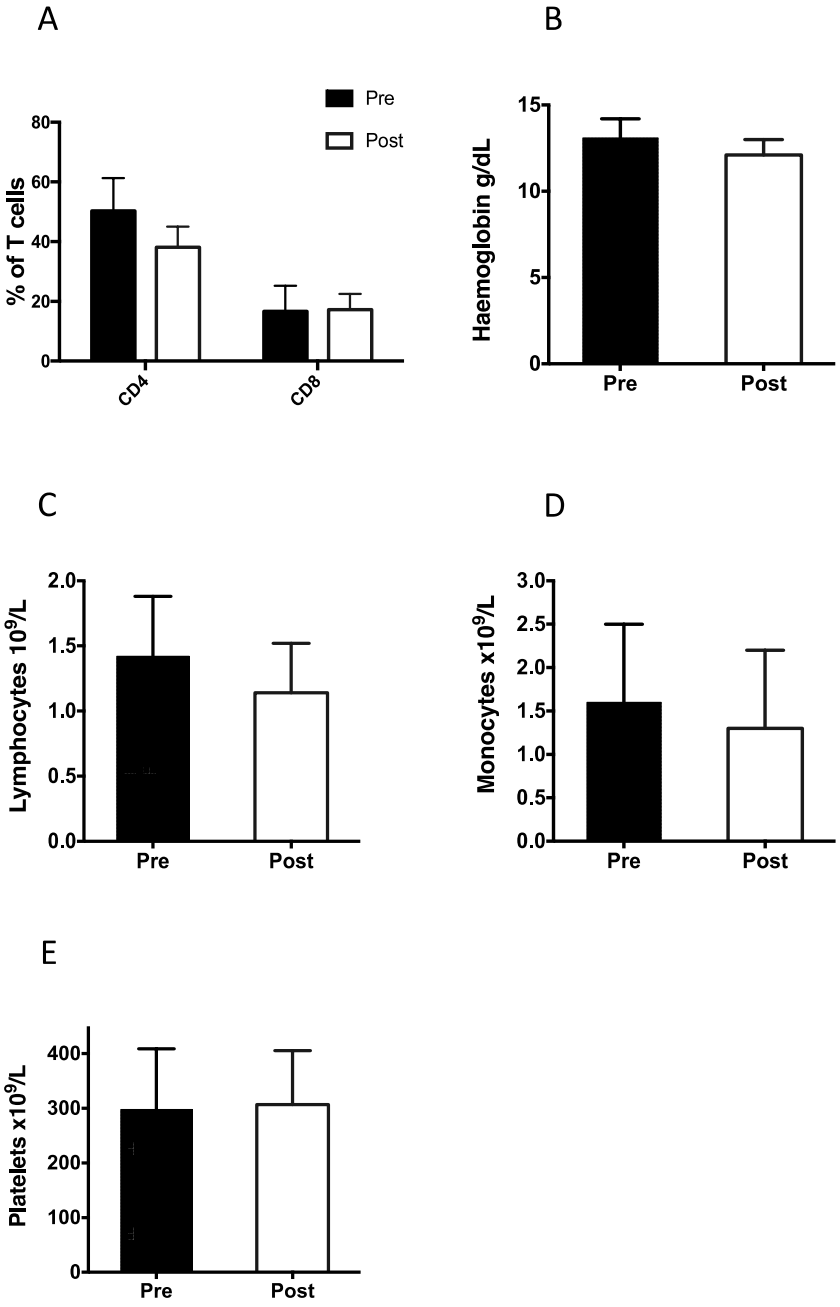


Figure 35A: Gating strategy to isolate $CD4^{+}CD127^{low/-}CD25^{+}$ T-cells (T-regulatory cells)

Flow Activated Cell Sorting (FACS) was undertaken using the FacsCalibur flow cytometer (BD Biosciences) and analysed with Flowjo software (Treestar, USA). Tregs (T-regulatory cells) were isolated from peripheral blood and the lymphocyte population was gated based on forward and side scatter (left upper plot), followed by gating all $CD4^{+}$ T-cells (right upper plot [CD4=FITC]). The cell populations were then sorted based on CD127low/− and CD25+ expression (left lower plot, $CD4^{+}CD127^{low/-}$ T-cell gated population [CD127= APC]; right lower plot, $CD4^{+}CD127^{low/-}CD25^{+}$ T-cell gated population [CD25=PE]).

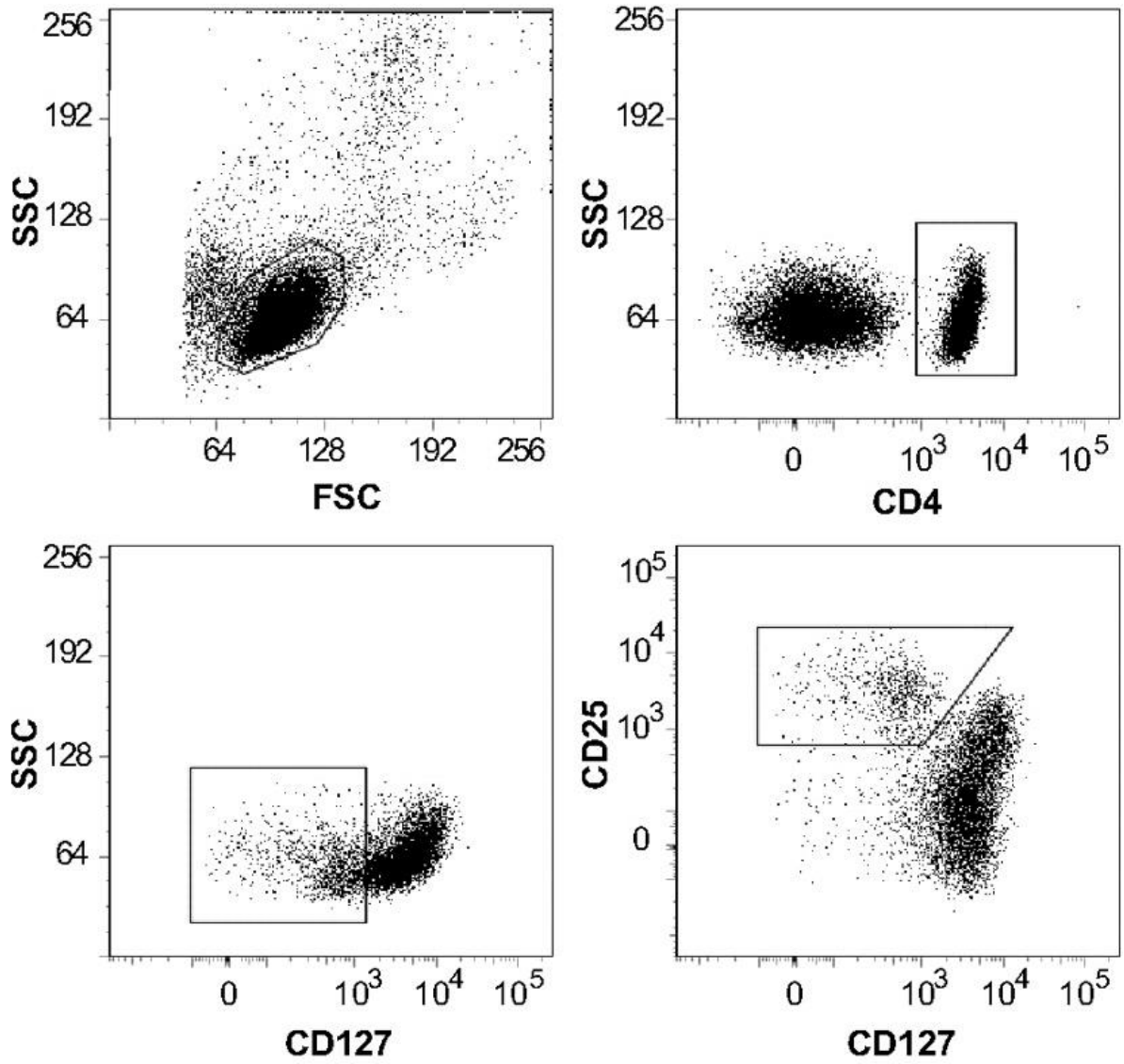


Figure 35B: Pre- and post-treatment analysis of Treg population (stratified by HPV status). Error bars show +/- standard error of the mean.

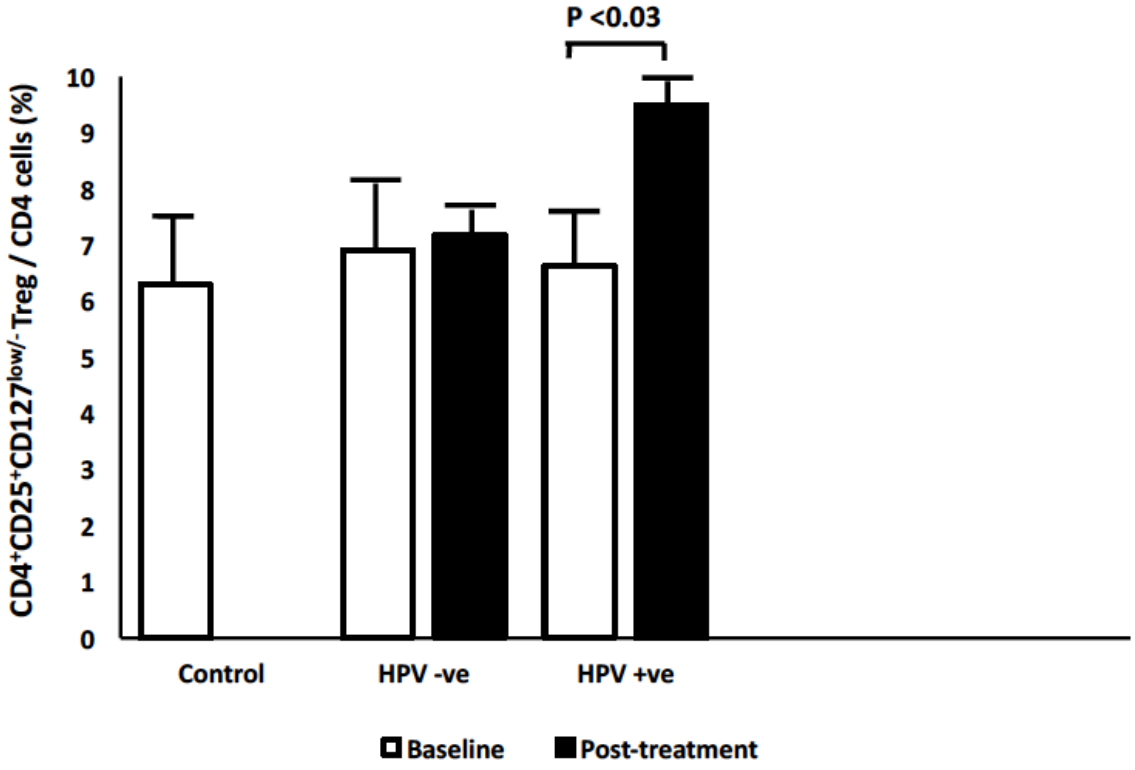
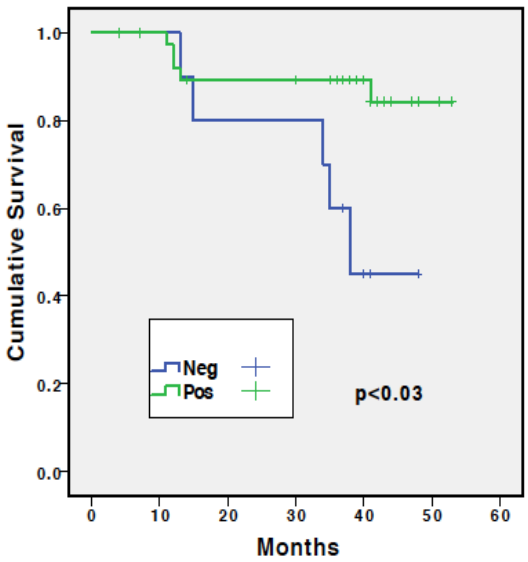


Figure 36: Disease Free Survival (DFS) stratified by a) HPV16 status and b) Post-treatment CD8 response to HPV16 E7 (Masterson *et al*., 2016a).

A multivariate model was developed using Cox regression to investigate the effect of clinical factors on disease free survival (HPV16, $\Delta CD4^+$ T-cell response to E6/E7 (pre-treatment versus post-treatment change in IFN-γ production), $\Delta CD8^+$ T-cell response to E6/E7 (pre-treatment versus post-treatment change in IFN-γ production), $p16^{INK4a}$, T_{reg} frequency (%), T stage, N stage, sex, physiological performance status, oropharyngeal subsite, histology grade, smoking, concurrent chemotherapy, age and $CD8^+$ / T_{reg} ratio (pre / post / Δ ratio).

A) HPV16



B) $CD8^+$ T cell response

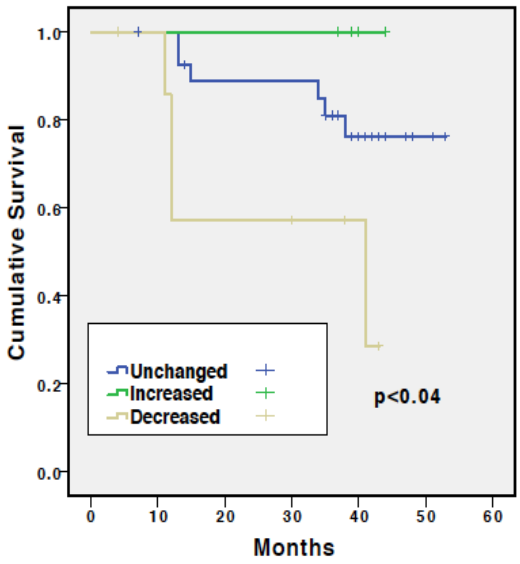


Figure 37: Hypothetical role for PD-1 (Programmed death protein 1) in HPV associated OPSCC (Masterson *et al.*, 2016a).

PD-1 is an inhibitory receptor expressed on various immune cells, including activated T-cells and T_{reg} cells. By blocking the interaction between PD-1 and its primary ligand, PD-L1 (red circle), T-cell effector functions are enhanced by increasing proliferation and cytokine activity (IFN-γ). Data from this study would suggest further work is required to discern the balance between cytotoxic and T_{reg} cells (before and after treatment).

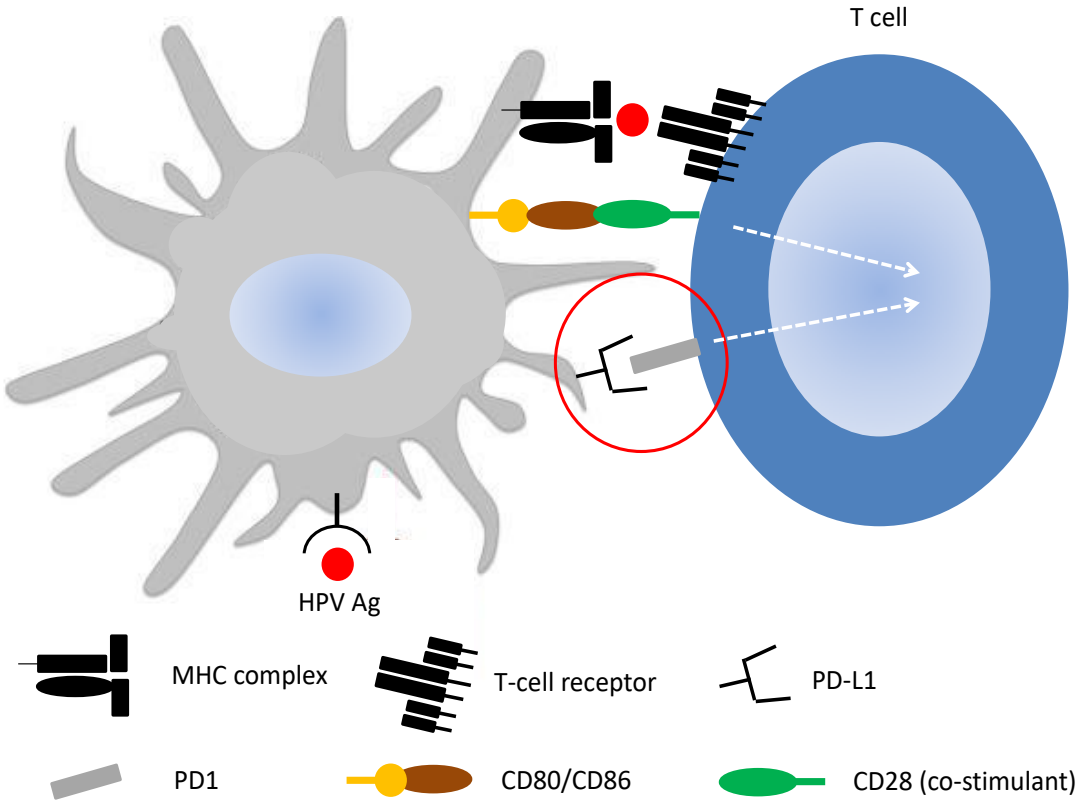
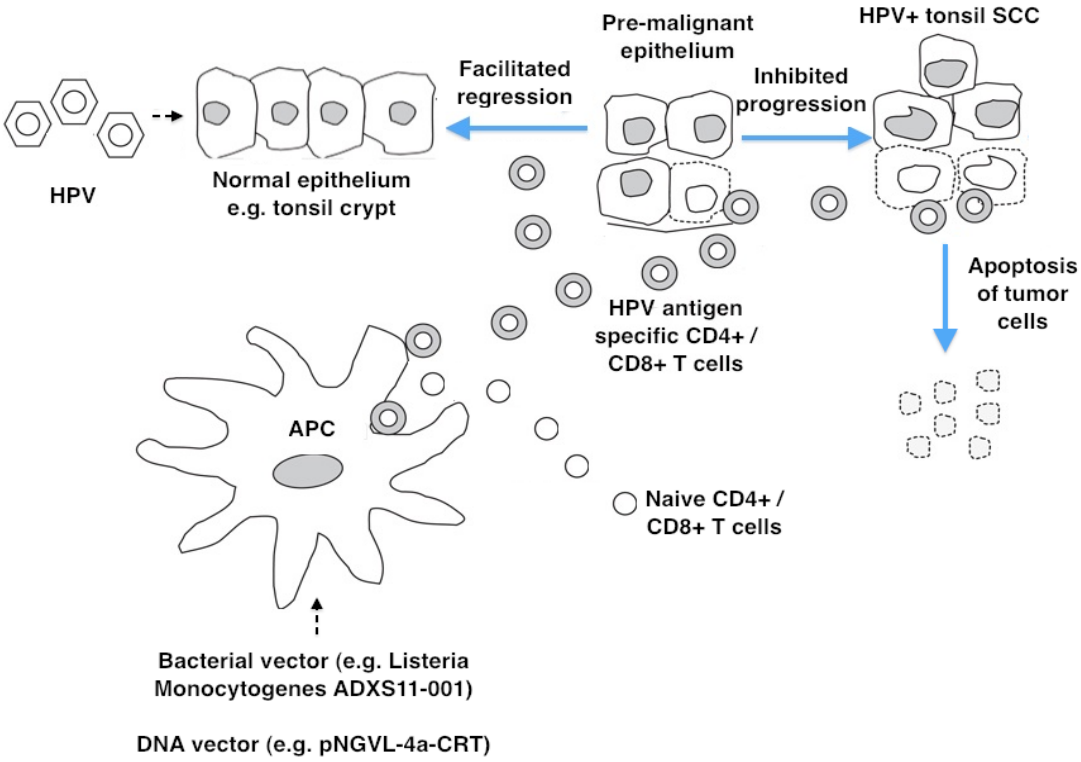


Figure 38: Therapeutic HPV vaccines (Masterson *et al*., 2014).

Immunotherapeutic approaches that target viral antigens can act systemically to target microscopic foci of malignant disease (local or distant) following chemoradiotherapy or tumour-ablative surgery. This may be of clinical benefit as the overall toxicity of tumour vaccines and other immune-based treatments are potentially less than that of chemoradiotherapy (with notable rare exceptions such as severe pneumonitis secondary to mTOR inhibitors e.g. Temserolimus [CCI 779]). Pre-clinical studies have utilized bacterial or DNA based vectors (e.g. Listeria Monocytogenes ADXS11-001, pNGVL-4a-CRT or GPI-0100) to deliver HPV16 E6/E7/L1 to Antigen Presenting Cells (APC) to augment T-cell-mediated immune response (Jones, 2012, Califano, 2011, Peng *et al*., 2015). The most prevalent approach utilises ADXS11-001 as an immunotherapy based on live attenuated *Listeria monocytogenes* that secretes fusion protein *Lm*-LLO-E7 targeting HPV-associated tumours. An attenuated version of the bacteria lacks PrfA, a transcription factor essential for the expression of virulence factors. This ensures the bacterium is unable to function as an intracellular parasite. The *Lm*-LLO-E7 protein is processed by the endogenous antigen-processing pathway, facilitating the presentation of epitopes in the context of MHC class I molecules (Cory and Chu, 2014). An alternative approach involves targeting the cellular protein p16INK4a (mixed with MONTANIDE ISA-51 VG), however this may have the potential to be non-specific in ~10% cases (Jäger E, 2011, Reuschenbach *et al*., 2015).



Chapter 5

Investigating the role of human papillomavirus and Epstein Barr virus in squamous cell carcinoma of the temporal bone

5.1 Introduction

Squamous cell carcinoma within the temporal bone region is a rare and destructive type of malignancy with a poor prognosis. The reported annual incidence is 1-6 cases per million, which accounts for 0.3% of all tumors of the head and neck (Gidley *et al*., 2010). Regional lymph node metastases can occur in 10-20% of patients but distant spread is rare (Sasaki, 2001, Moffat *et al*., 2005).

The site of origin of the invading tumour may not be obvious at presentation as the tumour progresses by direct incursion into the temporal bone and adjacent structures (parotid, temporomandibular joint, dura, brain). Multiple factors (Sasaki, 2001, Gidley *et al*., 2010) influence the development of SCC within the epithelium adjacent to the temporal bone. Exposure to ultraviolet light has been implicated in the development of SCC which originates in the pinna or external auditory canal (EAC) (Gidley *et al*., 2010). Chronic suppurative otitis media (CSOM) and radiotherapy have been widely reported to have an association (Moffat *et al*., 2005). SCC arising in the nasopharynx may also invade the temporal bone, by extension via the eustachian tube. One theory, which may connect the various sites and their predisposing factors, is the establishment of a chronic inflammatory process leading to metaplastic or neoplastic change (Lim *et al*., 2000).

Within the head and neck region, the association of HPV with SCC affecting the oropharynx, hypopharynx and nasopharynx has been clearly established. A recent meta-analysis of the world literature demonstrated that the proportion of oropharyngeal SCC caused by HPV has increased to 72.2% (Mehanna *et al*., 2012). New clinical trials are currently in progress to evaluate management based on this viral aetiology (Psyrri *et al*., 2012).

Epstein Barr Virus is also associated with various forms of cancer, particularly Hodgkin's lymphoma, nasopharyngeal carcinoma and Burkitt's lymphoma. The global burden of EBV associated cancer is lower than HPV with a prevalence ~2% versus ~5% respectively (Khan and Hashim, 2014, Forman *et al*., 2012). The potential contribution of viral infection to SCCs

of the whole intra-temporal region has not previously been investigated. This is surprising given the high morbidity and mortality associated with this disease and the established link to chronic inflammation. Case reports and small retrospective studies have indicated that HPV may play a role.

Jin *et al*. (1997) utilized PCR with consensus primers for the detection of HPV types 6, 11, 16, 18, 31, 33, 52b, and 58 in a retrospective study on FFPE material. HPV DNA ISH was performed, using probes to identify both low and high-risk HPV types. In fourteen cases with middle ear SCC of the temporal bone, eleven showed an association with high risk HPV activity.

Wang *et al*. (2009) reported presence of low risk HPV types in the external ear canal for 7 out of 10 cases with benign papilloma lesions <1cm in diameter. HPV ISH analysis was performed for the hybridisation of DNA probes, including both low-risk and high-risk HPV subtypes. The results revealed that seven out of ten cases were positive for low-risk HPV 6 or 11. No high-risk types (16, 18, 31, 33, 35, 39, 51, 52, 56, 58, and 66) were detected.

Miah *et al*. (2012) reported one case of malignant transformation of benign ear canal papillomatosis to SCC of the temporal bone. The study strongly encouraged further genetic analysis of benign lesions to assess their susceptibility for malignant transformation.

Marioni *et al*. (2003) analysed two patients with Schneiderian-type papilloma involving the temporal bone. Both samples had evidence of human papillomavirus using polymerase chain reaction with consensus primers.

If a viral aetiology for temporal bone SCC can be established beyond doubt, this would have important diagnostic, therapeutic and prognostic implications.

A review of the literature would suggest a possible association between high risk HPV and SCC of the temporal bone. Outstanding questions relate to corroboration of this data and assessment of clinical outcomes based on this viral aetiology.

This study will be based on a retrospective analysis of FFPE samples, such an approach is primarily based on the rare incidence of this carcinoma and the existing large number of bio-banked material already at this teaching hospital location.

One of the major limitations of this study relates to a decalcification technique utilizing trichloroacetic acid (TCA). TCA is known to have an adverse effect on the quality / amount of extracted DNA and as such this may reduce the sensitivity for high risk HPV subtypes.

The results described in the chapter have been published in Masterson *et al.* (2013), and some passages have been quoted verbatim from this publication.

5.2 Materials and Methods

5.2.1 Patients and Specimens

This study received formal approval by the National Research Ethics Service Committee of East of England (12/EE/44). Retrospective archival clinical samples were obtained covering the period 1983-2008 (Department of Histopathology, Cambridge University Hospital NHS Trust, UK). All cases with a diagnosis of SCC affecting the external ear canal or middle ear cavity were included. Biopsies were obtained at the time of the surgical procedure. Patient data including age, sex, smoking status, clinical presentation and disease stage (obtained from hospital and primary care records) were correlated to patient outcome (overall and disease specific survival).

5.2.2 Extraction of DNA

See section 2.1.2 DNA extraction (Temporal bone carcinoma samples (FFPE)).

5.2.3 Polymerase Chain Reaction

HPV PCR analysis

See section 2.2.2 PGMY PCR Analysis & 2.2.3 Nested PCR.

HPV DNA sequence analysis

See section 2.2.4 Direct cycle sequencing.

EBV PCR analysis

See section 2.2.6 EBV PCR.

5.2.4 *In situ* Hybridisation

See section 2.3.

5.2.5 Immunohistochemistry

See section 2.4. Primary antibodies were used at the following concentrations in PBS: p16 1:20 (BD Pharmingen), MCM7 1:200 (Abcam), Ki-67 1:150 and LMP1 1:150 (Dako, Glostrup, Denmark).

5.2.6 TP53 Sequencing

See section 2.2.7.

5.2.7 Statistics

Characteristics of the study population were summarised using descriptive statistics. Tumour samples were considered positive for HPV if detected by PCR (with DNA sequence analysis) or RNA *in situ* hybridization with corroborative evidence on p16 immunohistochemistry. Tumour samples were also considered to have an association with EBV if detected by PCR or LMP-1 immunohistochemistry.

Rates of overall and disease specific survival were estimated by means of the Kaplan–Meier method and were compared between these groups with the use of the log-rank test. The chi-squared test was used to analyse all other proportionate data between subgroups. Differences with values of $p<0.05$ were considered significant. All calculations were performed using SPSS® Version 17 (Illinois, USA).

5.3 Results

5.3.1 Clinical data

A total of 20 cases of temporal bone SCC involving the EAC or middle ear cavity were identified from the hospital records and entered the study (1 sample per patient). Most cases (70%) displayed moderately differentiated squamous cell carcinoma, the remaining cases were split equally between well and poorly differentiated carcinoma. The age at presentation varied from 37 to 79 years, with a median of 62.5 years (SD +/-11.3yrs). Eleven (55%) of the patients were men and nine were women. The mean follow-up period was for 34 months (range 1-184). Three-year disease specific survival was 40% (SE +/-11%) with no significant difference noted when stratified by HPV status, age, sex, smoking or disease stage. The clinical and laboratory data are summarised in **Table 15** and **Figure 39A+B**.

5.3.2 Detection of HPV by PCR

After three separate assays, most DNA samples (14/20) showed amplification of the β-globin 268 base pair (bp) product and were therefore deemed suitable for PCR evaluation of HPV. In all remaining samples (6/20), no further analyses were attempted.

DNA extracted from an HPV11 positive genital wart sample was used as a positive control for both β-globin and HPV PCR. One sample (case 4; **Table 15**) showed amplification of a 450bp product with PGMY primers. Nested PCR GP5+/GP6+ primer set showed a positive band (150 bp) in 3 patients (cases 2, 4 and 6; **Table 15**). DNA sequencing of positive samples revealed the subtype HPV16 in all cases. Point mutation analysis revealed minor heterogeneity to be present.

5.3.3 Detection of EBV by PCR

One patient sample (Case 19; **Table 15**) displayed evidence of EBV positivity when analysed by the EP_5 / EM_3 primer set. A control sample using genomic EBV DNA (EBNA–2) revealed the 182 bp amplicon.

5.3.4 *In situ* hybridisation

To determine transcriptional activity within the tumour tissue, samples noted to have β-globin amplification with PCR were subjected to further analysis by RNA *in situ* hybridisation. Two samples (each HPV+ on PCR / DNA sequence analysis) showed evidence of epithelial expression of HPV16 E6 or E7 and L1 mRNA. The control sense probe was negative. The positively stained cells were distributed throughout the entire layer of superficial neoplastic epithelium (**Figure 40A-D**). EBER RNA *in situ* hybridisation was utilised for the positive EBV sample but did not detect any evidence of active infection (**Figure 41A-D**).

5.3.5 Immunohistochemistry

All three HPV DNA-positive tumours underwent p16 immunohistochemistry, two of which displayed evidence of specific nuclear and cytoplasmic staining (**Figure 39B**). HPV DNA-negative controls did not display evidence of p16 activity. The LMP-1 immunohistochemical stain for detection of EBV was negative for all samples (**Table 15**).

5.3.6 TP53 mutation analysis

Sufficient DNA was available for direct sequencing of exons 5–9 of the TP53 gene in the majority (13/14) of samples within the study. A functional mutation was discovered in 3/10 HPV-negative and 0/3 HPV-positive samples (Table 15), however this difference was not statistically significant (p=0.28). All suspected mutations underwent forward and reverse primer sequencing.

5.4 Discussion

In addition to the findings of Jin *et al* (1997), this study suggests an association with high risk HPV in a minority of patients with temporal bone SCC. However, the mere presence of HPV types (as determined by both molecular and immunohistochemical techniques) does not imply causation for this disease. It follows a trend recently encountered in other areas of the head and neck (Ang *et al.*, 2010). Three out of 14 patients were found to have detectable HPV16 DNA. No significant difference in disease specific survival was detected for the HPV-positive group. All study patients were recruited sequentially and demographic details are similar to other reported case cohorts (Jin *et al.*, 1997, Sasaki, 2001, Gidley *et al.*, 2010).

Although the 21.5% HPV16 detection rate in this study represents a minority, the clinical significance may be apparent if duplicated by other centres and correlated to longer term outcomes. The study expands on similar research conducted by Jin *et al.* (1997), which was restricted to the middle ear cavity but nonetheless demonstrated high risk HPV16 in 11/14 samples. This higher HPV detection rate may reflect ethnic composition – this may be inferred based on the wide discrepancy between EBV seroprevalence rates in Taiwan (88.5%) when compared to Europe / USA (50-66.5%) (Balfour *et al.*, 2013, Dowd *et al.*, 2013). The above statistics for EBV may also reflect in the rate of nasopharyngeal carcinoma in Taiwan (16.9 / 100,000 person years) when compared to Europe / USA (0.5 -2 / 100,000 person years) (Chien *et al.*, 2001, Chang and Adami, 2006). A potential limitation of the Jin *et al.* (1997) study is the absence of clinical follow up and DNA sequence analysis to exclude contamination of samples.

A significant proportion of oropharyngeal cancers (40-75%) are now thought to have HPV16 DNA integrated within their genomic DNA, with minor contributions made by other oncogenic HPV subtypes (18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) (Ang K *et al.*, 2010). The rise in HPV-associated oropharyngeal carcinoma has been well documented (de Martel *et al.*, 2012) and it is noteworthy that all similar cases in the temporal bone region were identified in the last decade – this may reflect the quality of samples obtained or possibly the growing importance of HPV within the head and neck region.

In this cohort, the link with EBV is less evident, with only one patient demonstrating a possible association on PCR analysis. This may represent either the true level of background infection, inhibitory pathways *in vivo / vitro* or inadequate detection of a low viral load. The premise for investigating this virus stemmed from the proximity of the nasopharyngeal mucosa to the temporal region. EBV is associated with the overwhelming majority of nasopharyngeal carcinoma (NPC) as evidenced by expression of LMP1 or EBER in tumour cells (Young and Rickinson, 2004).

At present the definitive treatment for SCC of the temporal bone is with major surgery and free flap reconstruction with limited scope for primary medical therapy (Moffat *et al*., 2005). Previous studies will not have accounted for a potential viral aetiology which may confound clinical outcomes (Ang K *et al*., 2010). Although this malignancy is comparatively rare, the morbidity associated with current treatment would provide a strong argument for change. In common with other published datasets (Jin *et al*., 1997, Sasaki, 2001, Gidley *et al*., 2010), most cases in this study present with advanced stage IV disease. A practical application of this research may therefore involve targeted screening to ensure earlier diagnosis.

It is tempting to conclude from the survival analysis (**Figure 39A**) that HPV-associated patients showed a trend towards improved survival but small numbers prevent this from proving statistically significant. If this limited data can be confirmed on a larger scale this may support studies which observe that repression of E6 and E7 (in HPV-positive SCC) will lead to activation of the p53 and pRb pathways, decreased cellular proliferation and cellular growth arrest (Major *et al*., 2005). This situation is quite different from conventional disease (HPV-negative SCC), where an irreversible p53 mutation may be present (Oliver *et al*., 2002). It is of interest to note that all such mutations found in this study were restricted to the HPV-negative cohort, however, no clear divide is evident.

HPV is primarily transmitted through close skin or mucosal contact during vaginal, oral or anal sex (Cason and Mant, 2005). Research conducted recently in the United States indicates that, at any single time point, 42% of females may have an anogenital HPV infection, in comparison with <7% in the oral cavity region (Gillison *et al*., 2012). Genital-finger

transmission may be possible but has yet to be conclusively proven (Gottschling *et al*., 2009). One observational study recently investigated the presence HPV DNA on hand samples in patients with a prior diagnosis of penile HPV. Seventy percent of specimens were positive, the majority of which were of the same HPV type (Bosch *et al*., 2006).

Non-genital routes of HPV infection have yet to be fully elucidated but it is generally accepted that haematogenous spread is unlikely (Gottschling *et al*., 2009). It is also contentious as to whether HPV may undergo fomite transmission from person to person (e.g. towels, sheets, medical instruments) (Syrjänen and Puranen, 2000). Major *et al*. suggest that spread from mother to child (vertical or perinatal transmission) of HPV6 or HPV11 may rarely lead to juvenile onset recurrent respiratory papillomatosis (Major *et al*., 2005). However, Fredericks *et al*. (1993) suggest that HPV genotypes detected in exfoliated maternal cells can be traced in oropharyngeal mucosa of infants in up to 70% of cases. Smith *et al*. (1991) demonstrated the presence HPV DNA in ~3% of buccal mucosa samples in a cohort of 2 out 72 newborn infants. Cason *et al*. (1995) revealed HPV vertical transmission rates of ~73% at 24 hour after birth and demonstrated this persisted in ~80% of cases at 6 months.

So which method could potentially predispose to HPV infection within the temporal bone? The two obvious routes of ingress for the virus would include direct spread from the oral cavity through the eustachian tube or topical spread through the external ear canal. The presence of a tympanic membrane or mastoid cavity perforation will potentially influence either route.

Two of the three HPV-positive patients in this study had a pre-existing tympanic membrane defect; this was also a common finding in HPV-negative disease and prevents accurate localization of the primary to the external or middle ear cavity. **Figure 39B** suggests an association with viral positivity and extended length of otological symptoms that may support a link with chronic inflammation.

The exact prevalence of HPV16 at different sites within the head and neck region is at present undetermined. The best proxy marker comes from a systematic review by Gillison *et*

al. (2012), which demonstrated a 1% (95% CI 0.7-1.3) prevalence rate for oral HPV16 in the general adult population. A comparison with the SCC temporal bone group may suggest a significant difference (1% versus 21% $p<0.01$), however, this needs to be interpreted with caution due to the obvious baseline differences. The rate of oral cavity HPV persistence may also be higher than currently observed at the anogenital site, facilitating the potential for malignant change (Cason and Mant, 2005).

The discovery of human papillomavirus DNA from this cohort of samples is insufficient evidence to lead to the conclusion that HPV is involved in the causation of the disease. HPV is common on all skin or mucosal surfaces and the detection of HPV DNA may just be contamination. We therefore chose to investigate HPV transcriptional activity within the tumour site by RNA *in situ* hybridisation (**Figure 40A-D**). The detection of HPV16 E6 or E7 mRNA may indicate transcriptional carcinogenic activity and is considered the current standard for confirmation of HPV-associated disease (Schache *et al*., 2011).

Since HPV DNA in head and neck cancer is frequently integrated into the genome of host cells, it is of interest to detect HPV16 L1 mRNA in one of the patients. This may reflect capsid expression and active replication of HPV16; features more in keeping with an episomal status of the HPV genome. However, we note that L1 expression has also been detected in HPV-associated HPV HNSCCs affecting the oropharynx (Winder *et al*., 2009) and anogenital regions (Koncar *et al*., 2017).

The limitations of this study are disparate methods of sample collection, variable storage length and a decalcification technique utilising trichloroacetic acid (TCA). This study is restricted to the UK and may not reflect other countries with different age / ethnicity profiles. Finally, the sample size is limited due to the rare nature of this disease and therefore may not be applicable to the wider population.

This study outlines early clinical data that relate high risk HPV16 to SCC temporal bone and as such may have a direct impact on patient care. A theoretical cause for malignant transformation may be transmission of the virus through the eustachian tube or external ear canal in combination with longstanding inflammation. The data collected suggests the need

for further investigation, preferably in the form of a prospective setting. This will have the benefit of providing fresh biopsy material, which can improve quantitative analysis.

Table 15: Summary of clinical and laboratory analyses

Most DNA samples (14/20) showed adequate amplification of the β-globin 268 base pair product and were therefore deemed suitable for further analyses. All HPV-positive cases demonstrated wild type (WT) p53, in contrast, the three p53 mutations found were confined to the HPV-negative group.

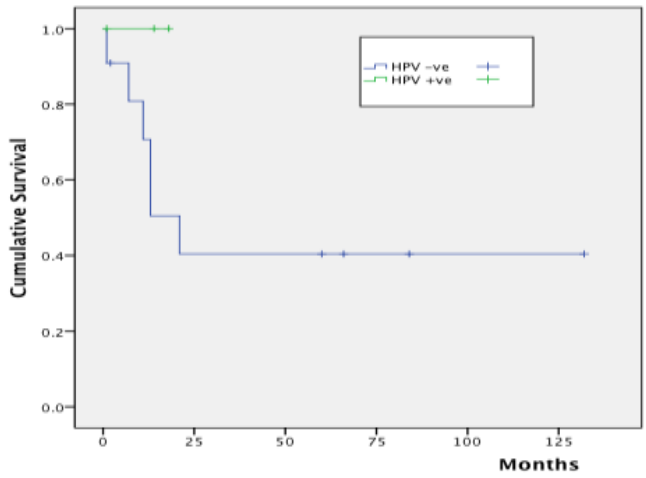
ID	Age (Yrs)	Date of Diag.	Sex	F/U (Mth)	Disease stage	Alive or Dead	SCC Histol.	EBV PCR	EBV LMP	ß-globin PCR	HPV PCR	HPV nest PCR	HPV16 E6/E7 mRNA	TP53 status
1	75	2008	M	11	IV	D	Mod	-	-	+	-	-	-	M
2	50	2011	F	23	II	A	Mod	-	-	+	-	+	-	WT
3	71	2009	M	11	IV	D	Mod	-	-	-	n/a	n/a	n/a	n/a
4	79	2011	M	<1	IV	D†	Mod	-	-	+	+	+	+	WT
5	53	2006	F	69	II	A	Mod	-	-	+	-	-	-	M
6	54	2011	F	19	IV	A	Mod	-	-	+	-	+	+	WT
7	49	1993	M	6	IV	D	Poor	-	-	-	n/a	n/a	n/a	n/a
8	71	2006	F	13	IV	D	Poor	-	-	+	-	-	-	WT
9	74	2006	M	65	IV	A	Mod	-	-	+	-	-	-	WT
10	53	2005	M	7	IV	A	Mod	-	-	+	-	-	-	WT
11	49	2003	F	7	IV	D	Mod	-	-	+	-	-	-	WT
12	67	2005	M	21	IV	D	Mod	-	-	+	-	-	-	WT
13	47	1997	F	13	IV	D	Mod	-	-	+	-	-	-	WT
14	62	1985	F	184	IV	A	Mod	-	-	-	n/a	n/a	n/a	n/a
15	64	1986	M	1	IV	D	Poor	-	-	+	-	-	-	WT
16	63	1983	M	132	IV	D†	Well	-	-	+	-	-	-	WT
17	69	1994	F	31	IV	D	Poor	-	-	-	n/a	n/a	n/a	n/a
18	68	1984	M	1	IV	D†	Well	-	-	-	n/a	n/a	n/a	n/a
19	56	1992	M	89	IV	A	Mod	+	-	+	-	-	-	M
20	37	1989	F	11	IV	D	Mod	-	-	-	n/a	n/a	n/a	n/a

M, male; F, female; SCC, squamous cell carcinoma; Mth, month; Diag, diagnosis; Histol, histology; Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated; n/a, not applicable; F/U, follow up duration; †= Non-malignant cause of death; WT, wild type; M, mutated.

Figure 39: Temporal bone carcinoma survival analysis (Masterson *et al*., 2013)

A, Disease Specific Survival stratified by HPV status (Log Rank *p*=0.211). **B**, HPV PCR positive sample clinical dataset.

A



O	N	Number of patients at risk			HPV
6	11	4	3	1	-ve
0	2	1	0	0	+ve

O = number of deaths; N = number of patients

B

ID	Otological symptoms	DNA sequence	p16	HPV16 RNA E4*	HPV16 RNA E6*	HPV16 RNA E7*	HPV16 RNA L1*
2	Otorrhea for 18 months. Previous radiotherapy to ipsilateral temporal lobe 13 years previously.	16	-	-	-	-	-
4	Mastoid cavity discharging >30 years. Recent otalgia >8 months.	16	+	-	-	+	+
6	Bilateral otitis externa >3 years. Six month history of blood stained discharge	16	+	-	+	-	+

*Positive = Anti-sense stain evident with suppression of sense control

Figure 40: HPV detection in SCC of temporal bone (Masterson *et al*., 2013).

A, HPV16 E7 mRNA sense probe (control) shows no uptake in epithelium. **B**, HPV16 E7 mRNA antisense probe shows increased uptake throughout all layers of the epithelium. **C**, HPV16 L1 mRNA sense probe (control). **D**, HPV16 L1 mRNA antisense probe also shows uptake throughout all layers of the epithelium.

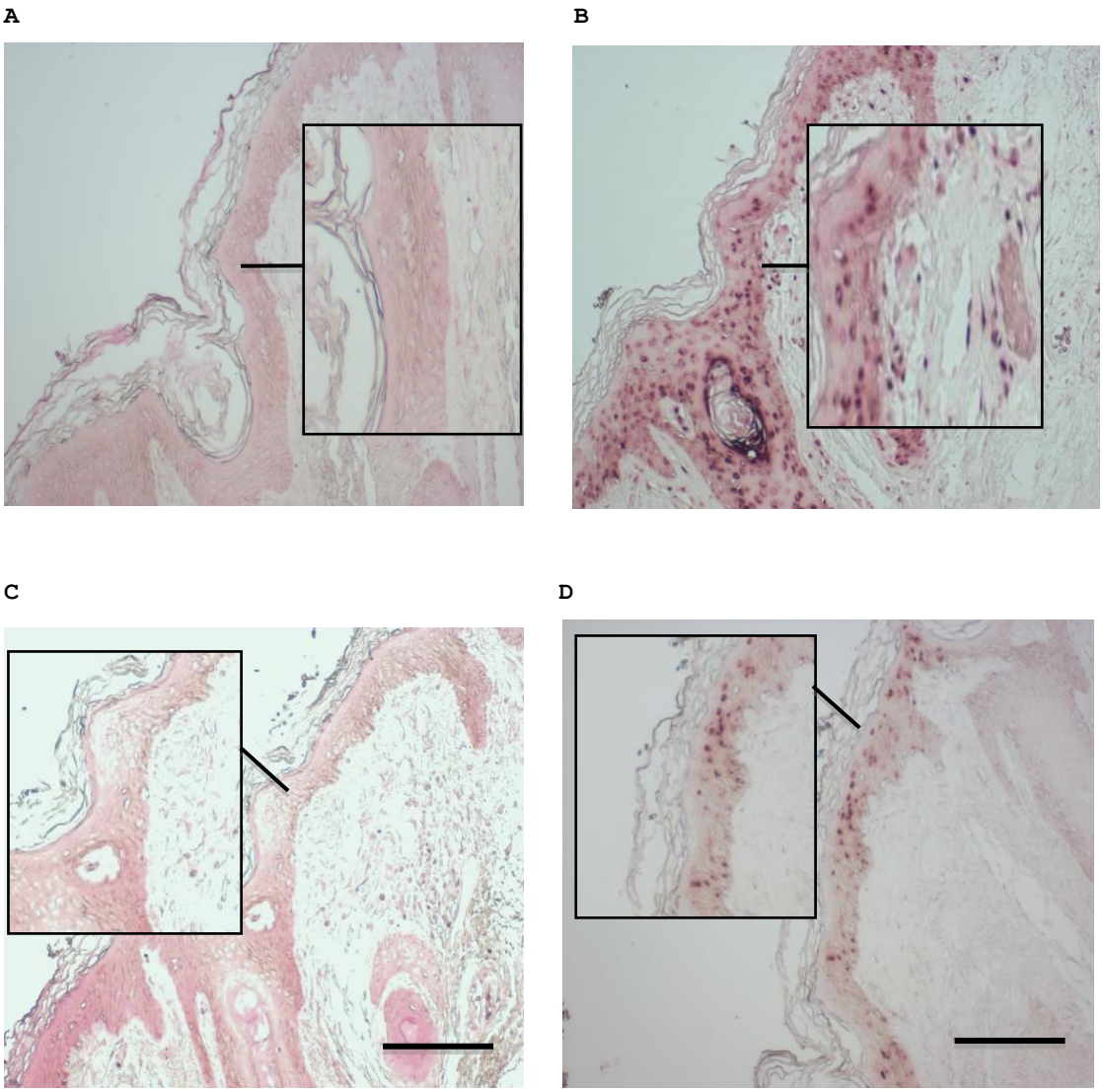
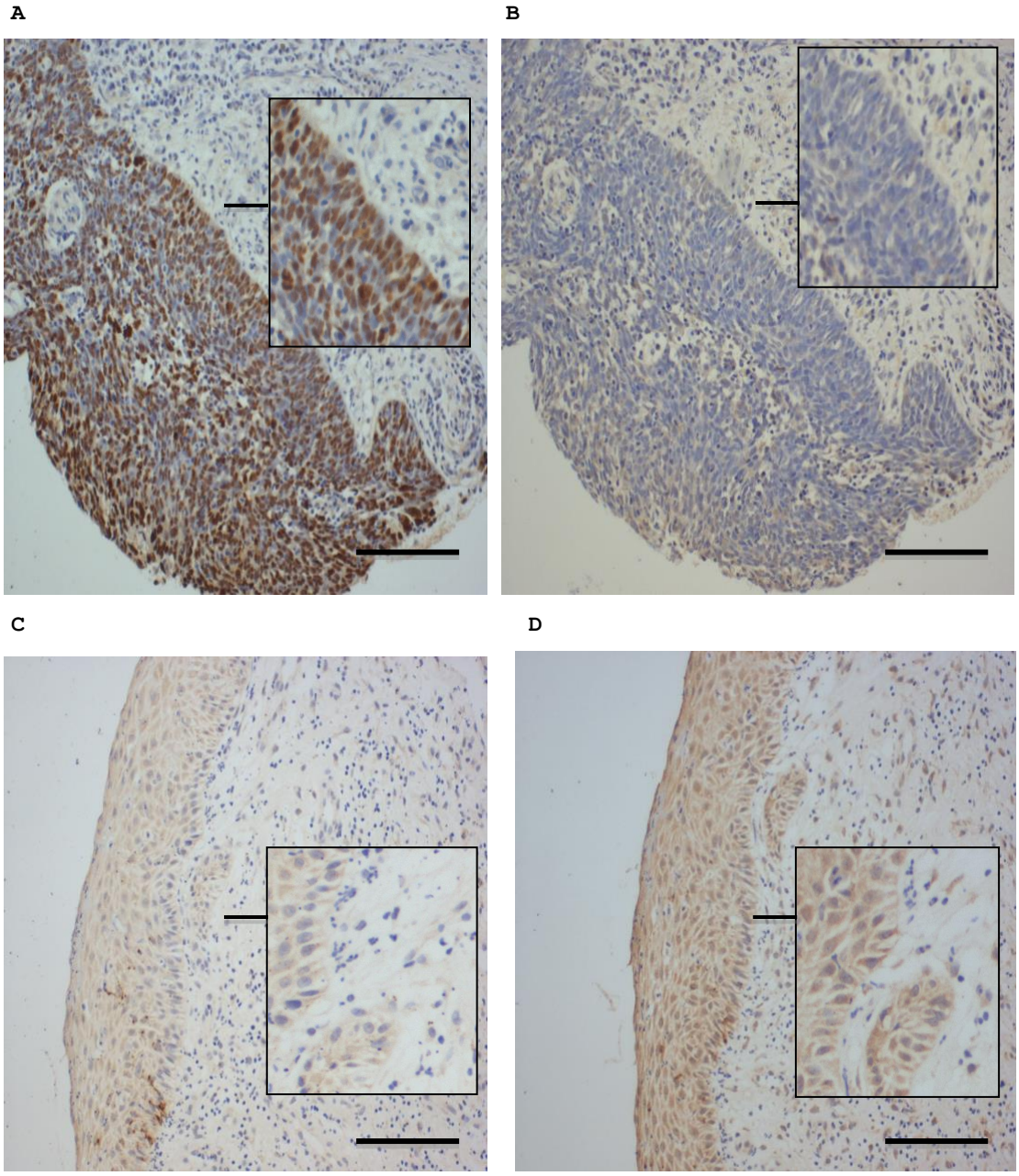


Figure 41: Epstein-Barr virus (EBV)-encoded small RNAs (EBERs) RNA in situ hybridisation (linear scale bar 200µM; inset picture magnified x2.5). **A**, Nasopharyngeal carcinoma control reveals strong uptake of probe in comparison to **B**, RNA negative probe. **C**, EBV PCR positive patient reveals no uptake of probe when compared to **D**, RNA negative probe (Masterson *et al*., 2013).*



*An RNA Negative Control Probe is a single oligonucleotide, designed from zebra fish DNA. Basic Local Alignment Search Tool (BLAST) analysis is utilized to confirm no homology with any human sequence.

Chapter 6

Discussion

The data presented in this thesis emphasize the significant influence of high risk HPV infection on HNSCC (and in particular OPSCC). Further studies are likely to focus on developing non-invasive screening tools, therapeutic strategies based on vaccination / immune modulation or de-escalation of current treatment protocols.

6.1 HPV and cellular changes in benign, pre-malignant and malignant oropharyngeal disease

The principle aim of this thesis was to investigate the hypothesis that dysplastic epithelial changes preceding the development of HPV-associated OPSCC can be identified by specific molecular and/or genetic abnormalities.

As the majority (~75%) of patients with HPV-associated OPSCC present at an advanced stage (Licitra *et al*., 2006, Ang *et al*., 2010), earlier detection of malignant disease could be of immense benefit.

The project focused on examination of OPSCC fresh frozen samples using whole transcriptome analysis to identify potential biomarkers of relevance. Verification of results by reverse transcription–quantitative PCR confirmed predictable fold changes of RNA expression in HPV-associated disease e.g. *CDKN2A* / *CCND1*. A testis-specific gene not normally expressed in somatic cells, synaptonemal complex protein 2 (*SYCP2),* showed consistently elevated fold change in malignant tissue when compared to normal tissue. In addition, secreted frizzled related protein 1 (*SFRP1*), a modulator of the Wnt signaling pathway was found to have significantly reduced expression in malignant tissue.

Laser capture microdissection on FFPE samples enabled specific investigation of regions identified as malignant or pre-malignant (if present). These confirmed the findings on fresh frozen tissue and in addition showed elevated expression of *SYCP*2 in pre-malignant tissue when compared to normal tissue. This latter finding indicates the potential for *SYCP2* as biomarker for screening purposes.

Future work

A significant finding of this work relates to increased expression of SYCP2 in HPV associated pre-malignant tissue (adjacent to invasive oropharyngeal carcinoma). If replicated on a larger basis, this may contribute to a potential non-invasive screening tool for early stage (pre-clinical) disease. A literature review would suggest testicular cell adhesion molecule 1 (TCAM1) and stromal antigen 3 (STAG3) are also potential candidate biomarkers in this respect and the possibility exists to combine multiple target transcripts into a single quantitative assay (Lambert *et al.*, 2015). Regarding SYCP2, further functional studies using animal models are required to elucidate a true causal relationship with HPV associated malignant disease. It could simply be the case that elevated expression of SYCP2 reflects genomic instability and de-differentiation of epithelial cells.

Prospective randomised controlled clinical trials with a post hoc analysis of pre-treatment biopsies have now established that detection of HPV in oropharyngeal tumours (a subgroup within the head and neck region) may confer a survival advantage to the patient (Fakhry *et al.*, 2008, Ang *et al.*, 2010). This recognition that HPV-positive tumours have a better prognosis than traditional tobacco- and alcohol-associated disease has particular importance with regards to current treatment protocols (Genden, 2013, Ferris, 2015). It leads to the question of whether it may be possible to safely de-escalate the intensive therapies (primarily aimed at the type of disease now known to be HPV-negative) that can unnecessarily harm this subgroup (Masterson *et al.*, 2014). One large multi-centre study based in the UK (PATHOS, 2015) shows promise by investigating the surgical management of patients with HPV-associated OPSCC. Adjuvant treatment protocols (ameliorated RT dose or removal of chemotherapy versus standard therapy) are then allocated according to TNM stage, smoking status or histopathological risk factors e.g. extra-capsular spread / positive surgical margins.

Within the oropharynx, HPV16 constitutes the overwhelming majority (~90-95%) of HPV subtypes associated with malignancy (Schache *et al.*, 2011). This contrasts with uterine cervix mucosa, where approximately 15 high-risk subtypes cause >99% of disease. These findings suggest that the cancer progenitor cell of origin is likely to influence the clinical outcome when HPV infection is involved, and that the natural history of HPV life cycle is

different according to the site and cell of infection. Indeed, there is some evidence to suggest that in OPSCC, the viral genome is episomal and not integrated, as in uterine cervix malignancy (Leemans *et al.*, 2011). The majority of studies in the literature, however, support the role of HPV integration in OPSCC (Leemans *et al.*, 2018).

So far, the largest single cohort of patients with HNSCC (n=279) subjected to whole genome sequence (WGS) analysis with phenotypic correlation is The Cancer Genome Atlas (TCGA, 2015). Using this example, head and neck clinicians in the UK are embarking on an ambitious goal, within the auspices of the 100K Genomes Project (Caulfield *et al.*, 2017), that will involve WGS analysis for >500 patients with HNSCC. Recruitment will primarily involve new HNSCC patients diagnosed at major oncology units, and in addition, appropriately stored samples from existing national clinical trials. The new project is likely to identify a broader range of biomarkers that can aid diagnosis, prognosis and facilitate targeted treatment - recruitment is due to finish in 2019 (Appendix 13).

6.2: T cell response to HPV E2, E6 and E7 in patients with OPSCC

A further aim of this thesis was to investigate the immunological response to treatment in HPV16 positive OPSCC. A subtle immune defect has long been thought to trigger the susceptibility of some individuals to persistent HPV infection with either low or high risk HPV types (Duray *et al.*, 2010). Such variations in host immune response may also affect the likelihood of HPV-related head and neck cancer. Cell-mediated immune responses to HPV16 E2, E6 and E7 were examined in peripheral blood using IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT) in patients with OPSCC treated by chemoradiotherapy.

T cell responses against E6 or E7 peptides correlated with HPV DNA/RNA status. Within the HPV16+ OPSCC cohort, enhanced immunoreactivity to antigen E7 was linked to improved survival. In addition, an observed increase in regulatory T cell frequencies (in the HPV

associated cohort only; see Figure 35 page 168) after treatment would suggest that immunosuppression may contribute to a reduced HPV-specific cell mediated response.

Future work

Although the E6 and E7 epitopes are apparently immunogenic in patients with HPV-associated oropharyngeal carcinoma, it is unclear why specific T-cells are unable to eliminate or prevent oncogenic change at an earlier stage. Further studies are required to explain this resistance of tumour targets to cytotoxic T cells and to find potential strategies that will increase the chances of developing HPV-based therapeutic vaccine in the future. In addition, clarification regards the elevated regulatory T cell frequencies observed in HPV associated OPSCC requires further prospective research – if confirmed this will also provide a potential useful therapeutic target.

The overall toxicity of tumour vaccines and other immune-based treatments are potentially much less than that of radiotherapy or chemotherapy (with notable rare exceptions such as severe pneumonitis secondary to mTOR inhibitors). Adjuvant immunotherapeutic approaches that systemically target viral antigens could have the potential to remove microscopic foci of malignant disease.

Research strategies have so far utilized HPV16 E6/E7/L1 as the target antigens for antigen presenting cells (APC) to augment T-cell-mediated immune response (Califano J, 2011, Jones, 2012, Peng *et al.*, 2015). Dendritic cells are a specific form of APC that can prime T cells *in vivo* to become cytotoxic T lymphocytes (e.g. HPV16 E6/E7 CD8+) or T helper cells (e.g. HPV16 E6/E7 CD4+). An alternative approach involves targeting the cellular protein $p16^{INK4a}$, however this may have the potential to be non-specific in ~10% cases (Jäger E, 2011, Reuschenbach *et al.*, 2015).

6.3 The role of human papillomavirus and Epstein Barr virus in squamous cell carcinoma of the temporal bone

A final aspect of this study was to determine the role of HPV and EBV in the pathogenesis of squamous cell carcinoma within the temporal bone region. This is an uncommon tumour which is normally preceded by a history of inflammation within the external auditory canal (EAC) or middle ear / mastoid cavities (Gidley *et al.*, 2010). Although HPV has been implicated in many head and neck malignancies, its role in SCC of the temporal bone has not been established. If a viral aetiology can be found, this may affect treatment strategies (Jin *et al.*, 1997). Patients were investigated for presence of high risk HPV types using polymerase chain reaction, RNA *in situ* hybridisation, DNA sequence analysis, and p16 immunohistochemistry. In addition, all specimens underwent separate investigation for the presence of latent and active Epstein-Barr virus infection or mutations within exons 5-9 of the TP53 gene. HPV16 DNA was detected in ~20% of the available cases, however, no significant difference in disease specific survival was noted for the papillomavirus positive group. Epstein-Barr virus was not detected and all TP53 mutations were restricted to the non-viral group as expected (as HPV+ induced malignancy is associated with reversible inhibition of p53 function and not irreversible mutation). This study outlines early clinical data that relate high risk HPV16 to SCC temporal bone and as such may have a direct impact on patient care. A theoretical cause for malignant transformation may be transmission of the virus through the eustachian tube or external ear canal in combination with longstanding inflammation.

Future work

The data collected suggests the need for further investigation, preferably in the form of a prospective setting. A twenty percent HPV positivity rate alone does not suggest definite association nor causality. A large multi-centre trial involving fresh tissue and sensitive / quantitative DNA or RNA detection would be the next logical approach.

Chapter 7

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Chapter 8

Appendices

Appendix 1

Full heatmap of 223 transcripts that are differentially expressed between HPV-positive and HPV-negative OPSCC tumors (p<0.01; FDR 0.5%). Heatmap continued next page.

Appendix 2

RT-qPCR data for **prospective** fresh tissue **A)** HPV16 positive (samples 1-18 peach background) and **B)** HPV16 negative (samples 19-24 blue background) cohorts. When the optimal primer concentration produced a linear response to input cDNA, samples were analyzed in triplicate for each tested transcript (CDKN2A, CRCT1, SYCP2, SFRP1, CRNN, DLG2). Beta-actin (ACTB) was utilized as the housekeeping gene. N, normal tissue; M, malignant tissue. Efficiency range 98-103%; Slope range 3.4 - 3.2; LLD, Lower Limit Detection range 0.01 – 0.001.

Appendix 3

RT-qPCR data for **retrospective** cohort (LCM invasive versus normal tissue; HPV16 positive samples 1-23 peach background; HPV16 negative samples 24-27 blue background). FFPE tissue samples were subjected to laser capture microdissection to enable RNA extraction from representative regions of invasive carcinoma and adjacent normal tissue.

Appendix 4

RT-qPCR data for **retrospective** cohort (LCM carcinoma in situ versus normal tissue; HPV16 positive samples 1-10 peach background; HPV16 negative samples 24-25 blue background). FFPE tissue samples were subjected to laser capture microdissection to enable RNA extraction from representative regions of in situ carcinoma (pre-malignant change) and adjacent normal tissue.

Appendix 5

Disease free survival - multivariable and univariable statistical analysis. A multivariable proportional hazards model using Cox regression analysis revealed HPV16, SYCP2, smoking and physiological performance status to have significant influence on DFS.

Appendix 6 & 7

Receiver Operating Characteristic (ROC) curve to evaluate prognostic accuracy of SYCP2 (Appendix 6) / SFRP1 (Appendix 7) biomarker expression in regions of HPV+ in situ carcinoma. All control subjects were patients undergoing tonsillectomy for benign pathology. A further analysis incorporated data from the HPV-negative OPSCC cohort.

Appendix 8: Data summarising HPV status and T cell response for each subgroup

FFPE samples: $p16^{INK4a}$ immunohistochemistry was performed on FFPE tissue using a mouse monoclonal antibody (BD Biosciences). DNA *in situ* hybridization consisted of a probe directed against high risk HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 (Ventana INFORM HPVIII, Tucson, Arizona). Genomic DNA was extracted from 9 x 3.5 μm FFPE sections using a QIAamp tissue kit in accordance with manufacturer's guidelines (QIAGEN Ltd, UK). DNA was eluted in autoclaved and nuclease free H_2O and stored at –20°C. Concentration and purity of DNA was assessed by spectrophotometry. Samples had an absorbance ratio (260/280 nm) in the range of 1.8–2.0, and were diluted with H_2O to a concentration of 1–25 ng/μl prior to PCR. L1 and E6/E7 DNA PCR were performed as for fresh tissue samples (below). *Fresh frozen samples:* Oropharyngeal fresh tissue samples (max. 25 mg) were DNA extracted using a protocol published from this unit (Winder et al., 2009). As previously described, L1 DNA PCR analysis of tumour DNA (50-100 ng) involved the PGMY09/11 primer set with all negative samples subjected to further amplification using GP5+/GP6+ primers (Gravitt *et al.*, 2000). DNA bands identified after agarose gel electrophoresis were excised, purified using QiaQuick Gel Extraction columns (QIAGEN Ltd, UK) and sequenced directly (Source Bioscience, Cambridge, UK). E6/E7 DNA and cDNA PCR analysis involved primers specific for HPV16 E6/E7 (Sotlar *et al.*, 2004). For all

fresh tissue biopsies, parallel FFPE samples enabled $p16^{INK4a}$ immunohistochemistry (as above). N/A, not available.

Appendix 9: HPV16 E2, E6 & E7 response detection by IFN-γ ELISPOT pre and post radical therapy ($CD4^+$ & $CD8^+$)

A response was considered positive if the average number of spot forming cells (SFC) in HPV antigen well was 2 standard deviations (SD) above the average of negative control. The frequency of cytokine secreting $CD4^+$ / $CD8^+$ / $CD56^+$ cells were then derived by the formula: number of spots/number of cells per well. For this study, we assume that increased staining of $CD4^+$ equates to $CD4^+$ T cells; we assume that increased staining of $CD8^+$ equates to $CD8^+$ T cells; we assume that increased staining of $CD56^+$ equates to NK cells.

Appendix 10: IFN-γ production by CD56 cells (NK cell population) was utilised to discern non-specific (innate) immune response within the clinical subgroups. No significant difference was detected for all patients pre- or post-treatment. SFC, Spot Forming Cells.

Appendix 11: Treg analysis for each study participant pre- and post- radical therapy. Treg population defined as CD4+CD25+CD127low/-. For HPV+ OPSCC cohort, 16/24 patients demonstrated a significant increase in the Treg levels after conclusion of primary RT/CRT.

Appendix 12: Disease free survival - multivariate and univariate statistical analysis

A proportional hazards model using Cox regression analysis revealed HPV16, $CD8^+$ response to HPV16 E7 and smoking to have significant influence on DFS.

Appendix 13: 100K Genomes Project (Head & Neck domain)

Open letter to all major head and neck units in the UK.