Human Papillomavirus Associated Oropharyngeal Squamous Cell Carcinoma: Genetic Aberrations Associated with Local and Distant Recurrence

A thesis submitted to the University of Dublin, Trinity College for the degree of

Doctor in Medicine (M.D.)

by

Dr Shane Brennan

Supervisor:

Prof. Orla Sheils

Vice-Provost

University of Dublin

Trinity College

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Declaration

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I declare that this thesis has been submitted from my own work.

Full informed consent was obtained from participants who were alive at the time of recruitment for this study.

Shane Brennan

Table of Contents

List of Figures

List of Tables

Summary

The Human Papillomavirus (HPV) is an oncogenic virus which is associated with the development of head and neck squamous cell carcinoma (HNSCC), predominantly within the oropharynx. Approximately 25% of oropharyngeal squamous cell carcinoma (OPSCC) cases worldwide are attributable to HPV infection, with an estimated 65% in the United States.

Transmission is via exposure during sexual contact, with distinctive anatomical features of the tonsils providing this organ with a predilection for infection by HPV. HPV proffers a favourable prognosis in the head and neck region, with better overall survival (OS) rates in contrast to its HPV negative counterparts. This has resulted in extensive research into de-intensifying therapies aiming to minimise the morbidity induced by standard concurrent chemo-radiation therapy (CRT) without compromising efficacy. Despite the favourable prognosis, cases of recurrence and/or metastasis of HPV positive HNSCC do occur, and are linked with poor outcomes.

HPV 16 is the most frequent genotype identified in HNSCC, yet there is limited research to date studying the impact of other HPV genotype with respect to OS. A similar situation pertains to genetic aberrations associated in those with HPV positive HNSCC who recur, with limited studies today highlighting mutations in TSC2, BRIP1, NBN, TACC3, NFE2l2, STK11, HRAS, PIK3R1, TP63, and FAT1 have been identified in recurrent HPV positive OPSCC.

Chapter 1 presents a background of HPV positive OPSCC, with emphasis on mechanisms of diagnosis, current treatment guidelines and knowledge base today in

terms of HPV genotypes and somatic mutations linked with greater risks of recurrence.

Chapter 2 illustrates baseline demographics attained from the patient cohort with HPV positive OPSCC. This chapter further illustrates the laboratory techniques utilised to conduct this research, including HPV genotyping by targeted next generation sequencing (NGS) and targeted somatic mutation analysis using single-molecule molecular inversion probe (smMIP) panels based NGS.

Chapter 3 details the HPV genotypes and somatic mutations detected in patients with HPV OPSCC with and without locoregional recurrence (LR) and/or distant metastasis (DM), with direct correlation to demographics including: age, gender, smoking status, alcohol consumption status, site of primary, clinical and pathological stage at diagnosis, treatment regimen received, disease free interval (DFI).

Chapter 4, discusses the significance of these findings in the context of the current literature, with a focus on future direction of research.

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Abbreviations

ADEPT	Adjuvant De-escalation, Extracapsular Spread, P16 Positive, Transoral trial
AJCC	American Joint Committee Cancer
AMPK	Adenosine Monophosphate-Activated Protein Kinase
AKT	Protein Kinase B
Bax	BCL2 Associated X, Apoptosis Regulator
bp	Base Pair
BRAF	v- rapidly accelerated fibrosarcoma murine sarcoma viral oncogene homolog B1
BRIP1	BRCA1 Interacting Protein C-Terminal Helicase 1
BUB1	Benzimidazoles 1 homolog beta (yeast)
BTF3	Basic Transcription Factor 3
CAP	College of American Pathologists
CDK	Cyclin-Dependent Kinase
CDK4/6	Cyclin-Dependent Kinase 4/6
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
cDNA	Complementary Deoxyribonucleic Acid
ch-TOG	Colonic, Hepatic-Tumour Overexpressed Gene
CIN	Cervical Intra-epithelial Neoplasia
CMD	Cancer Molecular Diagnostics
CPS	Combined Positive Score
CRT	Chemo-Radiation Therapy
CTLA-4	Cytotoxic T Lymphocyte associated Antigen 4
DNA	Deoxyribonucleic Acid

Dig1	Drosophila disc large tumour suppressor
DFS	Disease Free Survival
EGFR	Epidermal Growth Factor Receptor
EHNS-ESMO-ESTRO	European Head and Neck Society, the European Society for Medical Oncology, and the European Society for Radiotherapy and Oncology
EMA	European Medicines Agency
FBXW7	F-Box and WD Repeat Domain Containing 7
FDA	Food and Drug Administration
FFPE	Formalin Fixed Paraffin Embedded
GPCR	G-Protein Coupled Receptor
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
Gy	Gray
h	Hour
hADA3	Human Alteration/Deficiency in Activation 3
HCL	Hydrochloric Acid
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus
HR	High-Risk
HPV	Human Papillomavirus
HSIL	High grade Squamous Intra-epithelial lesion
H&E	Haematoxylin and Eosin
H_20	Water
IgG1	Immunoglobulin G1

IHC	Immunohistochemistry
ICI	Immune Checkpoint Inhibitor
INHANCE	International Head and Neck Cancer
ISH	In-Situ Hybridisation
JAK	Janus Kinase
kb	Kilobase
KDM6A	Lysine- Specific Demethylase 6A
KDM6B	Lysine- Specific Demethylase 6B
KMT2D	Lysine Methyltransferase 2D
LCR	Local Control Region
LKB1	Liver Kinase B1
LR	Locoregional Recurrence
LSIL	Low grade Squamous Intra-epithelial Lesion
MAPK	Mitogen Associated Protein Kinase
MCM	Minichromosome Maintenance
Min	Minute
mL	Millilitre
mmol	Millimole
MMP	Matrix-Metalloproteinase
mTOR	mammalian Target of Rapamycin
n	Number
NaOH	Sodium Hydroxide
NCCN	National Comprehensive Cancer Network
NCRI	National Cancer Registry of Ireland
NFE2L2	Nuclear Factor-Erythroid-Derived 2-Like 2

Nrf2	Nuclear Factor-Erythroid-2-Related Factor 2
ng	Nanogram
NGS	Next Generation Sequencing
ng/ml	Nanogram per millilitre
nM	Nanomolar
nmol	Nanomole
NBS1	Nijmegen Breakage Syndrome
NOS	Not Otherwise Stated
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)
OPSCC	Oropharyngeal Squamous Cell Carcinoma
ORF	Open Reading Frame
OS	Overall Survival
PATHOS	Primary surgical de-escalation trial in post-operative Adjuvant Treatment of HPV-pOsitive tumourS
PBM	PDZ-Binding Motif
PBM	PDZ-binding motif
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein - 1
PDGFRA	Platelet Derived Growth Factor Receptor A
PD-L1	Programmed Cell Death Ligand - 1
PDZ	Psd-95 (Post Synaptic Density Protein), DlgA (Drosophila Disc Large Tumour Suppressor) and ZO1 (Zonula Occludens-1 Protein)
PFS	Progression Free Survival
PILs	Patient Information leaflet
PIP2	phosphatidylinositol 4,5-bisphosphate

PIP3	Phosphatidylinositol 3, 4,5-bisphosphate
PI3K	Phosphoinositide 3 Kinase
pM	Picomolar
pRB	Retinoblastoma protein
PSD95	Post synaptic density protein 95
PTEN	Phosphatase and Tensin Homolog
P16 INK4a	P16
QC	Quality Control
qPCR	Quantitative Polymerase Chain Reaction
RCPATH	Royal College of Pathologists
RHEB	Ras Homolog Enriched in Brain
ROI	Region of Interest
RPL32	Ribosomal Protein L32
RPM	Revolutions Per Minute
RPS27A	Ribosomal Protein S27a
RT	Radiation Therapy
RTK	Receptor Tyrosine Kinase
RVEEH	Royal Victoria Eye and Ear Hospital
SCC	Squamous Cell Carcinoma
Sec	Second
SJH	Saint James's Hospital
smMIP	single-molecule Molecular Inversion Probe
STAT	Signal Transducer and Activator of Transcription
STK11	Serine Threonine Kinase 11
TACC3	Transforming Acidic Coiled-Coil containing Protein 3

TCGA	The Cancer Genome Atlas
TSC1	Tuberous Sclerosis Complex subunit 1
TSC2	Tuberous Sclerosis Complex subunit 2
TUH	Tallaght University Hospital
UBA52	Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1
UICC	Union for International Cancer Control
ZO-1	Zona occludens-1 protein
µL	Microlitre

Chapter 1

Introduction

1.1 Cancers of the Head and Neck

Cancers of the head and neck are now the sixth most common malignant neoplasm, with an annual global incidence estimated at over 550,000 [1]. There are varying aetiological factors attributed to the development of cancers of the head and neck including gender, current and past alcohol and smoking history, nutritional status, as well as occupation exposures [2-4].

1.1.1 Incidence and Epidemiology of Head and Neck Cancer

The mucosal lining of the upper aerodigestive tract is composed of stratified squamous epithelium; with the exception of the nasal cavity, which is lined by ciliated pseudostratified columnar respiratory epithelium [5, 6]. Thus, the predominant malignant neoplasms in the head and neck region are squamous cell carcinomas (SCCs), comprising over 90% [7]. The most recent report from the National Cancer Registry of Ireland (NCRI) published in 2019 highlights that 2.1% of invasive cancers in Ireland between 2017 and 2019 were from the mouth and pharynx [8].

Oncogenic Human Papillomavirus (HPV) is associated with the development of SCC of the anogenital (cervical, vaginal, vulvar, penile and anus) and the head and neck region [9]. HPV positive head and neck squamous cell carcinoma (HNSCC) are classically located within the oropharynx [10]. The oropharynx consists of the base of tongue, soft palate, uvula, lateral and posterior pharyngeal wall, tonsils, tonsillar fossa and tonsillar pillars *(Figure 1.1)* [11]. Further analysis has illustrated that carcinogenesis associated with HPV within the oropharynx is most prominent in the tonsil and base of tongue regions [12, 13].

The global incidence of HPV positive oropharyngeal squamous cell carcinomas (OPSCC) is increasing with estimates showing that 25% of OPSCC cases worldwide are attributable to HPV infection, in contrast to North America which has a higher prevalence of approximately 65% [14, 15].

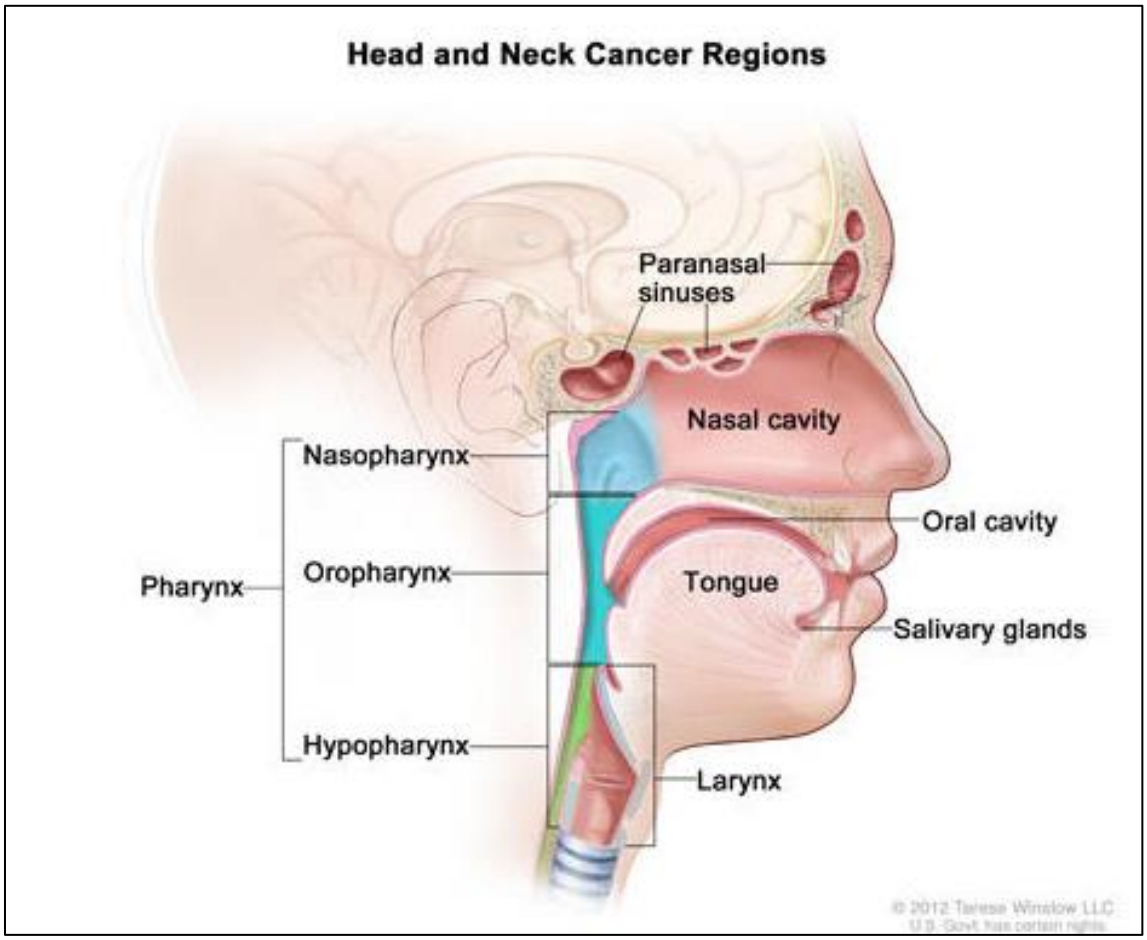

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Figure 1.1 *Anatomy of the Head and Neck region.*

Source: National Cancer Institute at the National Institute of Health, 2017 [16].

1.1.2 HPV Structure

The Papillomavirus structure is composed of a non-enveloped icosahedral capsid. The genome is comprised of double stranded Deoxy-Ribonucleic Acid (DNA) of 6 to 8 kilo-base-pairs [17]. The genome can be categorised into three parts: the long control region (LCR), the early region and the late region. The protein coding sequences of the genome are referred to as the open reading frame (ORF), which consists of the early and late region. The early region is composed of 8 ORFs; E1 – E8. E1 and E2 are core replication proteins, E4-E7, which are produced in oncogenic cells infected with the virus, contain the oncogenic properties [18]. Currently the roles of E3 and E8 are uncertain. The late region is composed of two ORFs: L1 and L2. L1 and L2 are the two structural proteins, forming the viral capsid. The capsid protein L1, is made up of 72 pentamers and the L2 protein, which comprises of approximately 72 pentamers [19]. *Table 1.1* illustrates the early and late genes of HPV and their respective functions.

Early Genes	Function
E1	Initiation of viral genome replication Origin of binding DNA helicase
E2	Transcription regulation – replication and maintenance of viral genome DNA binding activity E1 Helicase loader
E3	Unknown
E4	Destabilisation/remodelling of cytokeratin network Packing of viral particles
E5	Host cell transformation Inhibition of apoptosis in late events of HPV carcinogenesis stabilisation of EGFR and stimulation of MAPK activity
E6	Degradation of p53 Inhibition of apoptosis Induction of telomerase expression Interaction with PDZ domains involved in the maintenance of epithelial polarity
E7	Degradation of pRb Induction of DNA Damage Repair Upregulation of KDM6A and KDM6B
E8	Unknown
Late Genes	**Function**
L1	Minor capsid protein
L2	Major capsid protein

Table 1.1 *Early and late genes and their function. Table created using the following sources: Graham et al., McBride et al., Jr, J.S.L. et al. [20-22]*

Abbreviations: EGFR, Epidermal Growth Factor Receptor; MAPK, Mitogen-Activated Protein Kinase; pRB, Retinoblastoma protein; PDZ, Psd-95 (Post Synaptic Density Protein), DlgA (Drosophila Disc Large Tumour Suppressor) and ZO1 (Zonula Occludens-1 Protein); KDM6A, Lysine- Specific Demethylase 6A; KDM6B, Lysine- Specific Demethylase 6B.

1.1.3 Oncogenic Properties of the Human Papillomavirus

Stem cells are located at the basal layer of the stratified squamous epithelial lining the mucosa. Thus, the basal layer is where regeneration/replenishment of squamous cells occurs [23]. The work illustrated to date on the oncogenic properties of HPV derive from early work on cervical cancer tissue samples [24]. Based on modelling from virus entry into cervical squamous epithelium, the virus integrates into the host DNA [25].

As illustrated in *Figure 1.2*, the E proteins are expressed in the early stages of infection, thus E6 and E7 are located in the basal layers, where stem cells are located. E1, 2, 4, 5 are expressed within the supra-basal layers. Thus, oncogenesis is initiated in the lower regions of the stratified squamous mucosal layers. The structural late proteins are expressed as squamous cells mature towards the granular layer of the stratified squamous epithelium [26]. This is in keeping with the histological findings at microscopy, where early dysplastic changes are classically located in the basal regions with ascension as disease progresses [27, 28].

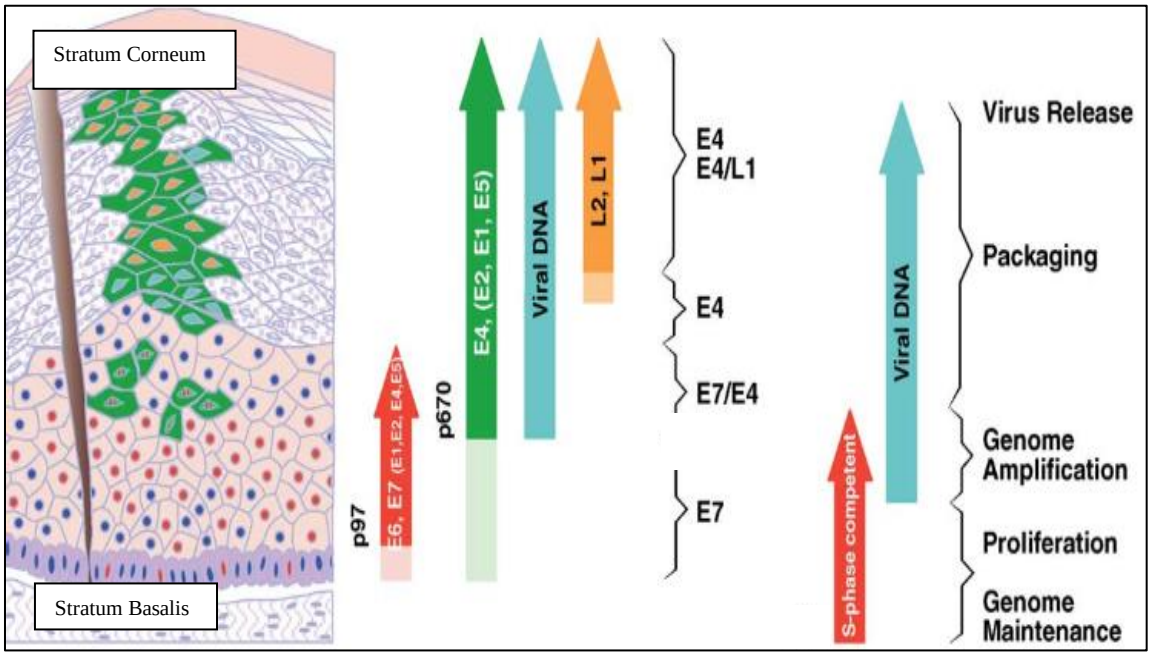

Figure 1.2 *HPV life cycle with highlighted protein expression.*

Source: Doorbar, J. et al. [28]

E6 and E7 proteins are associated with uncontrolled proliferation and carcinogenesis. Classically, proliferation of uninfected squamous epithelium at the basalis halts and only infected cells show further proliferation post the supra-basal layer. E6 and E7 promoter proteins ensure viral DNA is replicated and avoids the check points responsible for halting progression through the cell cycle or inducing apoptosis. Early promoter proteins such as p97 target E6, and late promoter proteins including p670 act on E7 [29].

Studies have shown that E6 and E7 are predominant in the early phase of the viral replicative cycle [24]. Both share oncogenic properties, which promote the inhibition of tumour suppressor genes [20-22]. E6 binds to cellular ubiquitin ligase E6-associated protein and the tumour suppressor gene p53, which results in a complex that degrades p53. E6 can also regulate p53 expression via interaction with the p300 and human alteration/deficiency in activation 3 (hADA3) trans-activators

[21, 24, 28, 30-34]. Furthermore, E6 upregulates expression of telomerase [35]. Once the cell has reached its Hayflick limit (the maximum number of times a cell can divide) it will die, thus each cell has a finite life span. If telomerase activity is upregulated, the length of the telomeres is maintained and cell death does not occur, thus permitting continued replication of damaged DNA [34]. In addition, E6 has been shown to block apoptotic pathways by downregulating pro-apoptotic proteins including Bak, Procapsase 8 and Fas associated death domain [34]. In summary, cells expressing E6 can evade mechanisms normally controlled by p53 including cell cycle check point controls and apoptosis [36].

Additionally, the E6 protein interacts and degrades Psd-95 (Post Synaptic Density Protein), DlgA (Drosophila Disc Large Tumour Suppressor) and ZO1 (Zonula Occludens-1 Protein) (PDZ) domain proteins that are associated with cell adhesion, cell cytoskeleton structure and apicobasal polarity [24, 34]. PDZ complexes play a role in cell cycle regulation via interaction with E6 proteins [24, 34]. The E6 proteins contain a PDZ-binding motif (PBM), which is located within the carboxyl terminus of the PDZ domain. This site is the point at which the E6 protein can interact with a multitude of proteins that possess PBMs [37-39]. This interaction invariably leads to their degradation and interrupts normal cellular processes.

E7 is associated with removing pRb from the transcriptional complex containing E2F transcription factor. Binding of E7 to the hypo-phosphorylated Rb results in the release of E2F. Thus, cell cycle activity progresses from G1 to S, permitting replication of altered DNA. E7 also blocks Cyclin Dependent Kinase (CDK) inhibitors p21 and p27, which regulate cell proliferation [21, 24, 28, 30-32]. E7 mediated breakdown of pRB proteins results in the overexpression of cyclin dependent kinase inhibitor p16 INK4a (p16). The is retained as the cell progresses

through G1 to S phase of the cell cycle [40]. The impact of the E6 and E7 oncoproteins on the cell are illustrated in *Figure 1.3.*

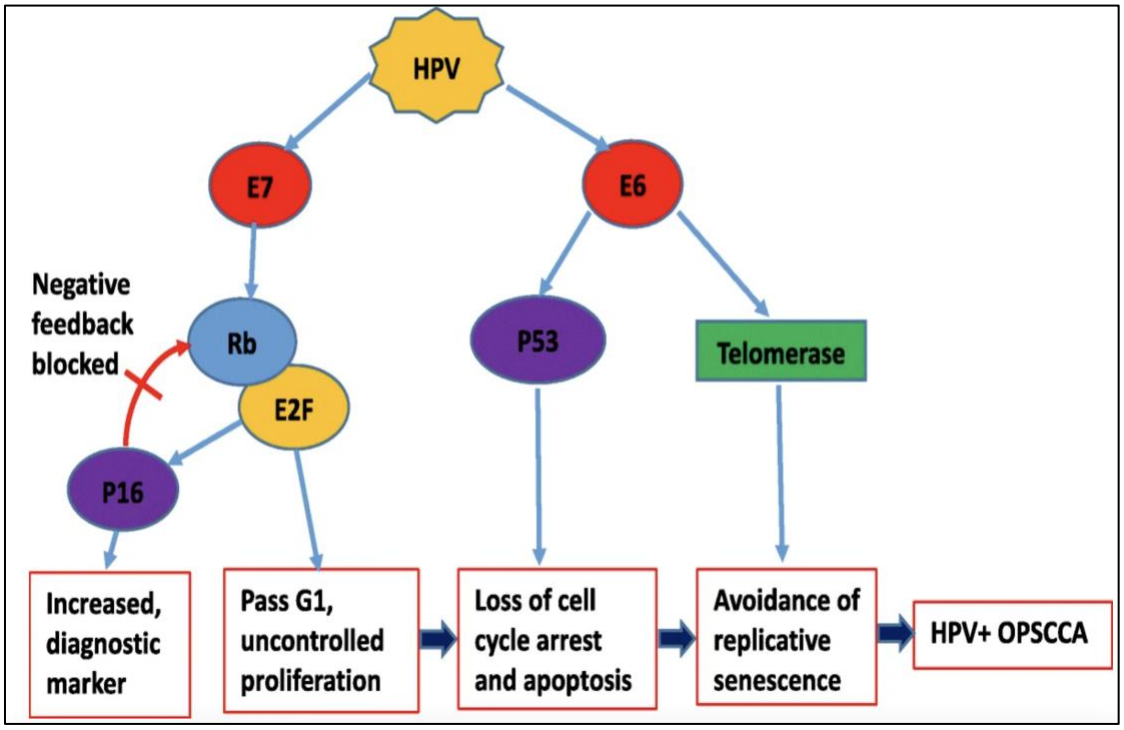

Figure 1.3 *The impact of E6 and E7 oncoproteins in the pathogenesis of HPV induced carcinoma.*

Source: Liu, C et al. [41]

Abbreviations: OPSCAA, Oropharyngeal squamous cell carcinoma.

1.1.4 HPV Transmission

The epitheliotropic oncogenic HPV is transmitted via skin-to-skin contact, skin-to-mucosa contact, or mucosa-to-mucosa contact. HPV transmission is thus predominantly via sexual behaviour including oral and vaginal sex, and oral-anal contact [42]. The changing dynamic of increased sexual partners and changes in common practices of sexual behaviour, is believed to have led to the increased

prevalence of HPV and thus the increasing incidence of HPV driven OPSCC [42]. A large-scale multi-centre study conducted by the International Head and Neck Cancer (INHANCE) consortium reported an increased risk of base of tongue and tonsillar cancer for individuals with a history of greater than five sexual partners, greater than three oral sexual partners and an earlier age at sexual debut [43]. In addition, a two-fold increased risk with one to five lifetime oral sexual partners and a fivefold increase if greater than seven oral sexual partners have been shown [44]. Despite this, other studies have reported that between 8-40% of individuals diagnosed with HPV positive HNSCC reported never having oral sexual contacts, with the majority in this cohort being white males [44-46]. Further supporting this, a study in 2013 described no 'significant' correlation between oral HPV infection and oral sexual contact [47, 48]. In conclusion, although contraction of HPV is accepted to be via sexual contact, other methods of contraction must exist.

1.1.5 Predilection for the Oropharynx

It may be perceived unusual that the tonsils, a lymphoid organ, would be a site for viral entry given the role of lymphocytes in immune response. However, there are numerous factors illustrating why the oropharynx, in particular the tonsil, is a prime location of HPV entry.

Within the oropharynx, the tonsillar crypts of the palatine and lingual tonsils are the most frequent site for HPV positive OPSCC [49-52]. The exact reasoning behind this predilection has not been elucidated. Within the uterine cervix, it is theorised that HPV transmission is facilitated by micro-abrasions of the squamous lining mucosa with exposure of the underlying basement membrane. This permits entry of

the virus to the basal region squamous epithelium, where stem cells are localised [52, 53]. The perceived mechanism for transmission in the head and neck region is thought to be different, and it is the specific anatomical and histologic structure of the tonsil that deems it to be the favoured site for HPV infection.

Firstly, the tonsils are composed of extensive tonsillar crypts, which increase the mucosal surface area. Secondly, the tonsillar surface is lined with non-keratinizing squamous epithelium, however within the crypts it is lined by reticulated squamous epithelium. This reticulated squamous epithelium has an intervening desmosomal network providing a scaffold-like structure linking the individual cells, thus making the layer penetrable and porous [25, 51]. This provides a mechanism for HPV to migrate to the basal layer of the reticulated epithelium [25, 50-52].

A further structural component distinct to the tonsil is an anomaly of the basement membrane. Underlying most epithelial structures exists a continuous basement membrane as a further protective barrier. The tonsillar basal epithelial layer is separated from the underlying lymphoid tissue of the tonsil by a discontinuous basement membrane, facilitating viral transmission [52].

In addition, the oncogenic virus has the ability to evade the immune system at this site [53]. Programmed Death Ligand 1 (PD-L1) acts as a checkpoint regulator ensuring control of the adaptive immune system [54]. However, in the general population, PD-L1 expression by squamous epithelial cells within the tonsillar crypts is elevated, in contrast to those on the surface [54]. PD-L1 interacts with Programmed Death-1 (PD-1) receptors on T-lymphocytes with resultant inhibition of its activity. The downstream effect is the evasion of the immune response [55]. In conclusion, elevated PD-L1 levels at the tonsillar crypts lead to reduced lymphocytic activity, permitting virus entry [25, 56, 57].

1.1.6 HPV Life Cycle

Once transmission occurs, based on modelling from virus entry into cervical squamous epithelium, the virus integrates into the host DNA with resultant dysregulation of E1 and E2 expression [25]. This results in the upregulation of the E6 and E7 proteins [58-60]. Although it is known that HPV-associated cancers contain integrated viral-DNA, viral-DNA inclusion into the host genome is not essential or universally observed amongst all HPV related cancers [61-63]. Viral-DNA can exist in an episomal form within the cell or mixed episomal and integrated, referred to as viral-human hybrid episome. This mechanism is illustrated in *Figure 1.4* [25, 63-65]. If present in this extrachromosomal/episomal form, the viral DNA will acquire genetic aberrations resulting in dysregulated E6 and E7 oncoprotein gene expression [62]. The prevalence of episomes that are not integrated into the host genome is far greater in HPV positive OPSCC than cervical SCCs [25, 62]. The implications pertaining to each of the various viral DNA constructs in the host have yet to be clarified.

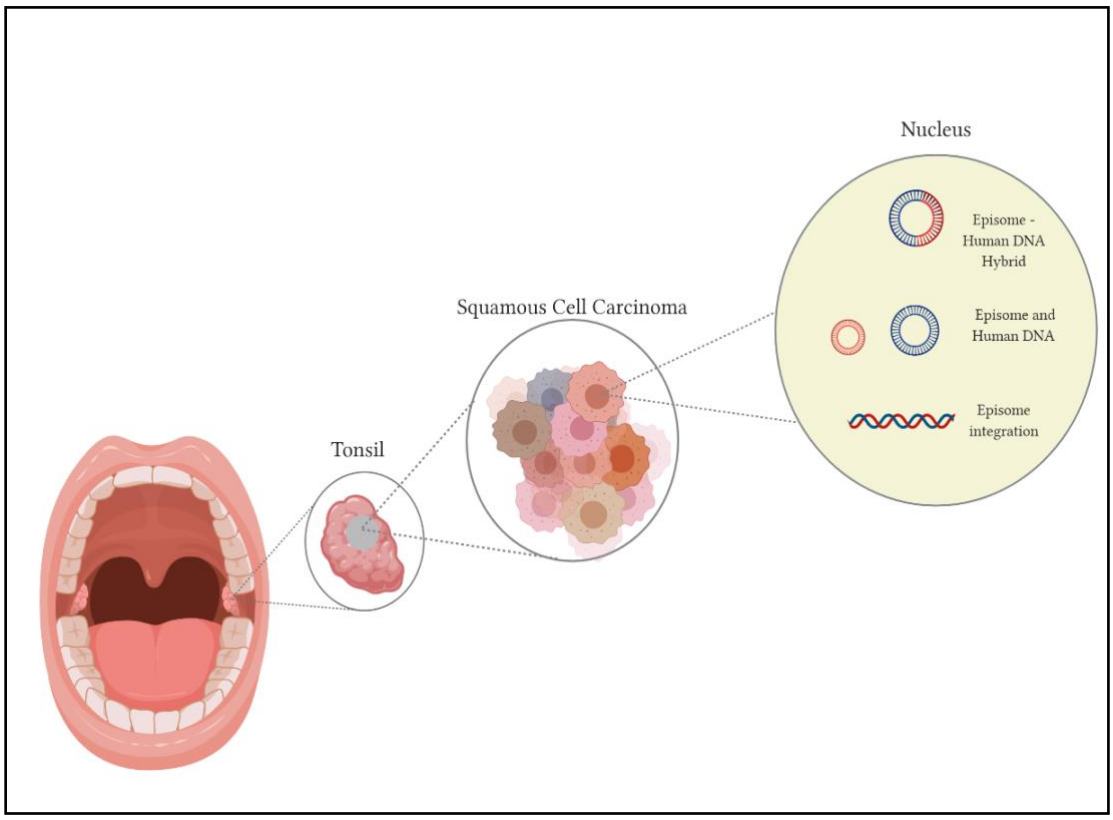

Figure 1.4 *Human Papillomavirus transmission into the host nucleus can result in (i) Viral DNA integration, (ii) a hybrid of conjoined viral-DNA and host DNA, (iii) independent viral-DNA, not integrated or linked with the host DNA.*

Source: Brennan et al. [66]

1.1.7 Pathogenesis of Squamous Cell Carcinomas

A well delineated stepwise progression from initial infection with HPV, to the development of premalignant dysplastic changes with resultant invasive SCC of the cervix, is accompanied by gross features identifiable during colposcopy and distinctive cytological and histological findings [60]. Infection with the oncogenic virus leads to evolving dysplastic changes, resulting in characteristic histological features classified by the percentage of epithelial layer involvement. Cervical intra-epithelial neoplasia (CIN) 1 low grade squamous intra-epithelial lesion (LSIL)

denotes lower third basal layer displaying dysplastic changes. CIN 2 and CIN 3 are collectively referred to as high grade squamous intra-epithelial lesion (HSIL), showing two thirds, or full thickness dysplasia [67]. The dysplastic findings identified on microscopy correlate to the protein level of E6 and E7 [27]. For example, in CIN 1, high levels of E6 and E7 are noted in the lower third of the squamous epithelium thickness. Above this point, in the upper two thirds, mRNA expression of E1, E2 and E4 has been identified [27, 59, 60]. Genetic alterations resulting in the upregulation of the CDKN2A, which has a key role in regulating cell cycle progression, has been identified in CIN 1 [59]. Further upregulation of Cyclin B1, budding uninhibited by benzimidazoles 1 homolog beta (yeast) (BUB1B) and minichromosome maintenance (MCM), all involved in mitosis and DNA replication, have been identified in CIN 2/3 [59, 68]. Upregulation of Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) has been identified in a minority of CIN 3 and a high proportion of invasive carcinomas [69]. Thus, as a sequence of genetic alterations which occur in conjunction with distinct histopathological features in the development of HPV driven cervical SCCs are detected by the screening Cytopathologist which permits the utilisation of screening techniques for premalignant lesions.

Regarding the head and neck region, in 1996 Califano *et al.* published a seminal research paper on the evolutionary genetic sequence of events for HNSCC, irrespective of HPV status [70]. The study documented genetic alterations and chromosomal changes that corresponded to histological findings, denoting a sequence of changes from normal mucosa to dysplasia, carcinoma *in-situ*, and finally invasive carcinoma. Loss of heterogenicity at 9p21, resulted in CDKN2A inactivation with subsequent transition from normal mucosa to hyperplasia [53].

This inciting event has been followed by mutations in *TP53*, with resultant histological evidence of transition from epithelial hyperplasia to dysplasia [53]. Furthermore, research to date has established that the genetic and chromosomal aberrations associated with cancer development occurs sequentially and are impactful based on their accumulative abnormalities rather than any individual mutation [52, 53, 70].

However, the caveat and limitations of the model of tumorigenesis described is that it pertains to the head and neck region in general. It is not specific for the oropharynx, nor does it take into consideration HPV status. *CDKN2A* and *TP53* mutations are drivers of HPV positive cervical carcinoma, with their identification in premalignant CIN lesions [59, 71, 72]. Thus, it could be postulated that mutations identified may all be related to HPV driven tumorigenesis. However, both are described in HPV negative HNSCC [53].

Precursor lesions with an oncogenic potential have been identified within the head and neck region. These 'white lesions' are referred to as leucoplakias, with only 5 to 25% showing dysplasia, and an annual rate of transformation to a malignant lesion of approximately 2-3% [73, 74]. It is estimated that HPV detection in leucoplakias whether dysplastic or non-dysplastic varies from 0 to 50% [75-78]. The proliferative verrucous variant of oral leucoplakias is most commonly associated with HPV positivity, most predominantly the HPV 16 genotype [77]. Although these premalignant lesions have a link to HPV, they are not identified within the oropharynx [79].

In conclusion, there is no identifiable premalignant lesion directly linked to OPSCC, in contrast to that of cervical cancer. However, HPV is deemed to have a role in the development of premalignant oral lesions. The lack of a premalignant

oropharyngeal lesion has limited research into the underlying pathogenesis in the area.

1.1.8 HPV Diagnosis

To confirm a diagnosis, a biopsy or resection specimen is required. There are some characteristic features identifiable on haematoxylin and eosin (H&E) staining that are indicative of HPV positivity. HPV positive HNSCC has a distinctive basaloid morphologic appearance (*Figure 1.5*), with a lobular growth pattern, infiltrating lymphocytes, no overlying dysplasia identifiable on the surface epithelium, and minimal if any keratinisation [44]. Note however, that HPV cannot be definitively diagnosed based on these histological features of H&E staining alone.

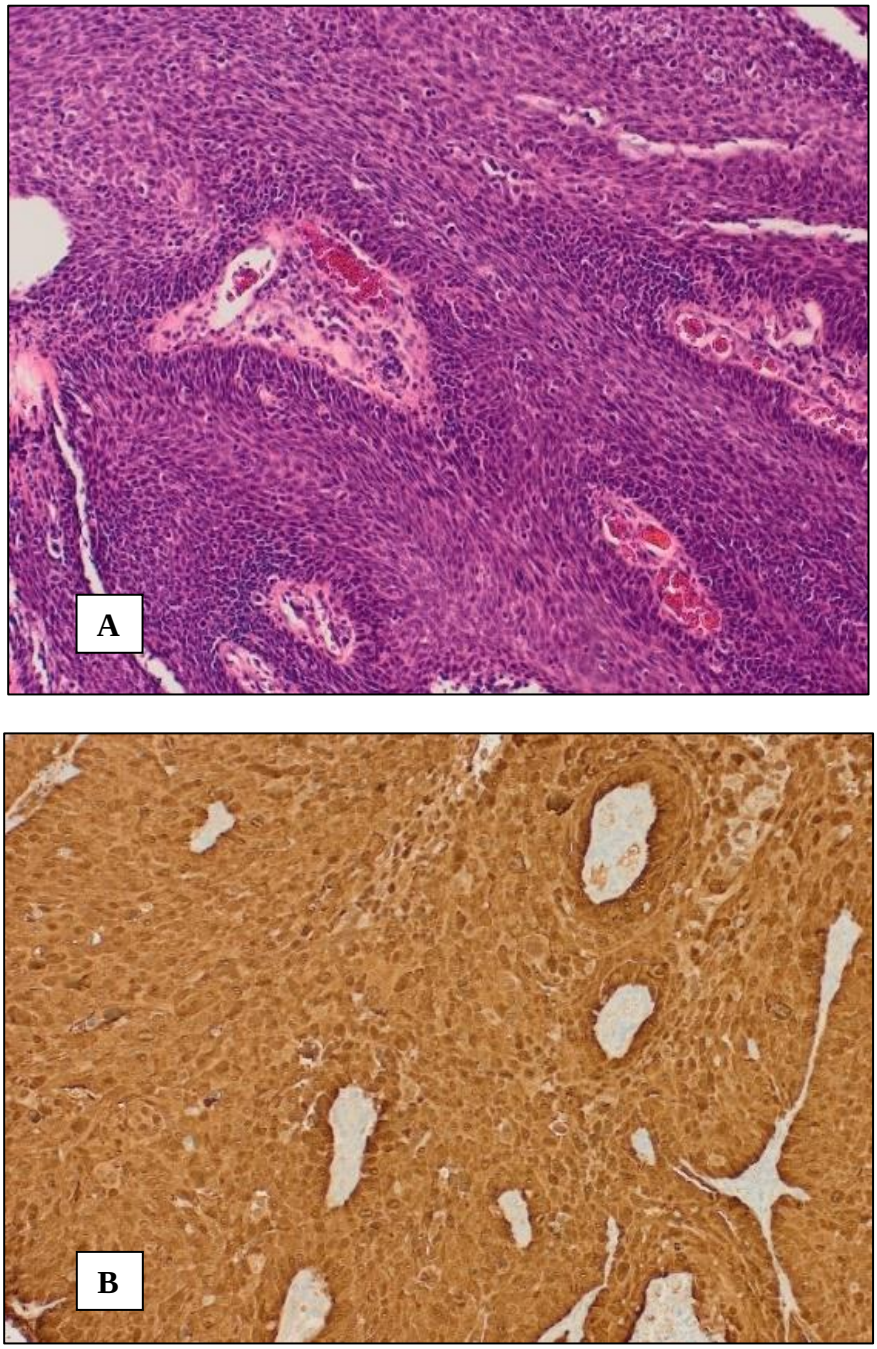

Figure 1.5 *(A) H&E stain showing non-keratinising invasive squamous cell carcinoma from the oropharynx (20X Magnification) (B) P16 immunohistochemical staining showing diffuse block nuclear and cytoplasmic staining of >70% from an oropharyngeal squamous cell carcinoma. (20X Magnification).*

There are numerous techniques utilised within the laboratory to detect HPV including Immunohistochemistry (IHC), DNA and RNA *in-situ* hybridisation (ISH) and polymerase chain reaction (PCR). The most utilised mechanism for the detection of HPV in Histopathology laboratories is p16 staining via IHC.

The expression of the tumour suppressor protein p16, is utilised as a key surrogate marker of transcriptionally active HR-HPV infection for many laboratories on histological specimens. It is the most common technique used to detect HPV in routine practice. As a mechanism for detection of HPV, it has a sensitivity of 94 – 97%%, a specificity of 83 - 86%, and false positive rates ranging from 3.8 to 7.3% [80-84].

p16 is encoded by the *CDKN2A* gene, located on chromosome 9p21.3. p16 binds to and inhibits *CDK4/6*, and thus phosphorylation of Rb protein cannot occur. If transcriptionally active HPV is present, the E7 oncoprotein binds to hypo-phosphorylated Rb protein, resulting in Rb degradation. After this, there is continued sustained activation of the E2F transcription factor leading to cell cycle progression and p16 over-expression in tumour cells [85-88].

As noted above, p16 analysis is undertaken using IHC with interpretation by a Histopathologist. p16 has characteristic nuclear and cytoplasmic staining, illustrated in *Figure 1.5*. The definition of what is accepted as positive staining with p16 IHC varies depending on which guidelines are followed. The Royal College of Pathologists (RCPATH) guidelines confer a positive diagnosis if there is “strong nuclear and cytoplasmic staining in >70% of malignant cells” [89] . The College of American Pathologists (CAP) defines positivity as “at least 70% nuclear and cytoplasmic expression with at least moderate to strong intensity” for p16 [22].

Alternate techniques to determine p16 levels include PCR using constructed consensus primers to target DNA sequences within the ORF of the L1 gene of HPV [86]. Consensus primers are readily available and the most frequently utilised include the following: MY09/11 which targets a 450-base pair (bp) sequence, GP5+/GP6+ which targets a 150 base pairs (bp) sequence and SPF10 which targets a 65 bp sequence[90-93].

In general, standard PCR has a high sensitivity but low specificity. As a tool for determining HPV positivity it cannot distinguish between integrated and episomal DNA, and between HPV positivity in neoplastic versus non-neoplastic cells [94]. A metanalysis of 8 studies found an average sensitivity of 98% and specificity of 84% [84]. HPV E6 and E7 mRNA detection by RT-PCR is considered by many as the gold standard for classifying an HNSCC as positive for transcriptionally active HR-HPV [86, 95, 96]. This method involves utilising an RNA template to produce complementary DNA (cDNA) via reverse transcriptase. In the HPV setting, HPV E6 and E7 mRNA provides the RNA template [86]. This method provides a measure of the viral load. It also has the added benefit of being able to distinguish whether the HPV DNA is integrated into the host genome or remains episomal. E2 is a regulatory gene, which is frequently disrupted when viral integration occurs in the host genome. Thus, PCR amplification of E2 fails if integration has occurred [94]. However, laboratory detection of HR-HPV requires a method that is sensitive, specific, amenable to be conducted on various types of histological/cytological samples, time efficient and cost effective to everyday diagnostics in Histopathology Departments. Thus, the frequent utilisation of p16 IHC is evident in hospital laboratories.

Another method using ISH, provides a precise localisation of a specific DNA sequence and it is the only method, which detects HPV within the nucleus [94]. The original ISH method utilised DNA-ISH, which has been shown to have a high specificity but low sensitivity with higher viral load copy numbers required to obtain adequate signal amplification [97]. However, with advances in RISH research, specific probes have been designed, which are complementary to E6/E7 mRNA [97, 98]. This method has been shown to have a greater sensitivity than DNA-ISH, as it is believed that there are a greater number of E6 and E7 RNA transcripts, which results in higher signal amplification [97]. Regarding DNA-ISH, studies in the literature have shown it has a lower sensitivity (57% to 92.7%) and specificity (81% to 100%) than IHC, with false negative rates for ISH ranging from 7.4% to 41% [81, 84, 94, 99-102].

1.1.9 HPV Genotype within the Head and Neck Region

The predominant HPV genus linked with OPSCC is the alpha papillomavirus [103, 104]. Further categorization of the alpha HPV genus is based on the oncogenic risk, extracted from work conducted predominantly on the anogenital region [105]. HPV genotypes are described as high risk if the prospect of developing a carcinoma is significantly more common than in individuals with HPV 6; which has been utilised as the pinnacle for low risk [106, 107]. Low risk HPV genotypes include HPV 6 and 11. HR- HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 [80, 103, 108-113].

Of note, both the beta and gamma genera have been isolated from oral cavity samples [114-117]. Studies have linked beta1 HPV 5, gamma 11 HPV and gamma

12 HPV with an increased risk of HNSCC, encompassing non-oropharyngeal regions of the head and neck [103, 115, 117, 118]. However, it has been extrapolated that the mechanism by which these particular non-alpha HPV genera propagate neoplastic development is comparable to the mechanism driving cutaneous HPV positive SCCs [118]. To date there is limited research concentrating on genotypes outside of the alpha genus in HNSCC, and OPSCC pathogenesis. HPV 16 is the most prevalent HPV genotype in various studies of the head and neck region, identified in 70 to 90% of cases [110, 119-123]. Within the oropharynx, the HPV 16 genotype accounts for 84 to 88% of cases [45, 110, 124].

It is worth noting that not all studies discriminate findings relating solely to the oropharynx but combine figures from all regions of the head and neck. The prevalence of HPV 18 ranged from 0.6 % to 4 % [110, 112, 125-127]. HPV 33 and HPV 35 both account for 4.5% [110, 119, 126, 128, 129]. The less commonly identified genotypes include HPV 31 (0. 3 -1.06%), HPV 39 (0.8-4.3%), HPV 26 (1.7%), HPV 52 (1-2.7%), HPV 53 (0.3%), HPV 56 (0.25%), HPV 45 (0.5-1.4%), and HPV 58 (0.6 -5.8%) [45, 110, 119, 124, 130-132]. A full illustration of the prevalent HPV genotypes identified within the head and neck region is highlighted in *Figure 1.6*.

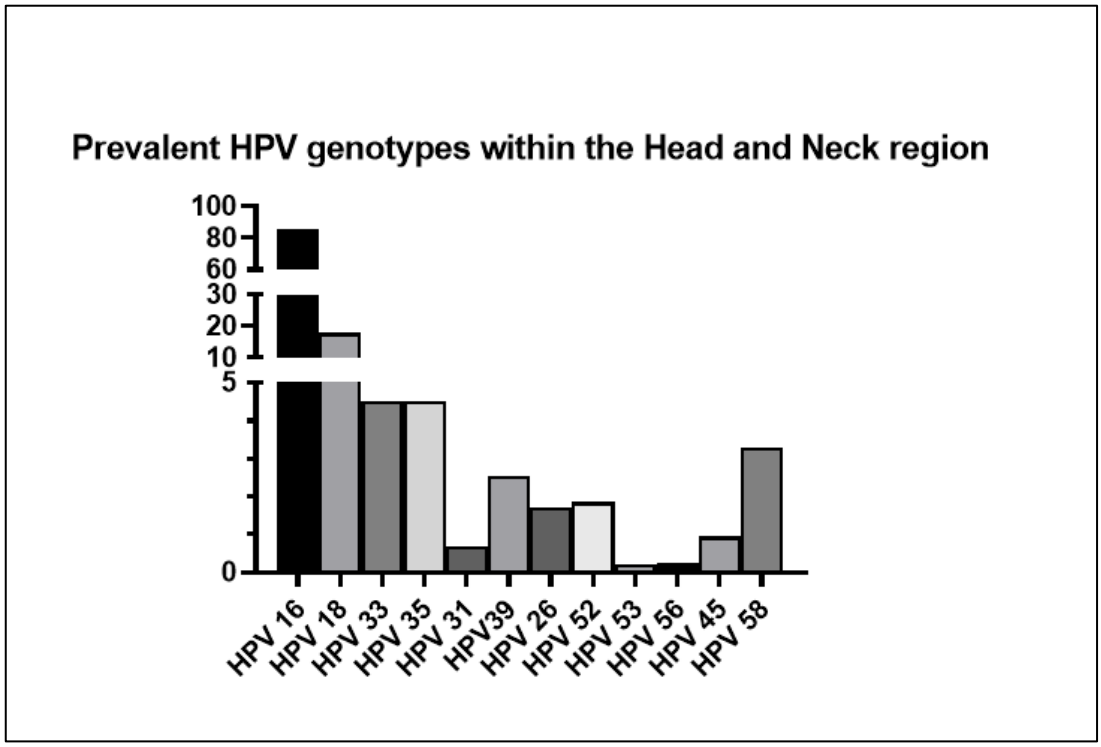

Figure 1.6 *Prevalent HPV genotypes within the Head and Neck region. Data sourced from the cited studies [45, 110, 119-124, 126, 128-133]. The x axis denotes percentage of prevalence.*

Source: Brennan et al. [66]

Comparing the head and neck region figures to those in the cervix, a retrospective cross-sectional worldwide study reviewing HPV genotypes of invasive cervical carcinomas highlighted that HPV 16 accounted for 61% of cases (HPV 18 (10%), HPV 33 (4%), HPV 35 (2%), HPV 39 (2%), HPV 45 (6%), HPV 53 (<1%), HPV 56 (<1%) [134]. An interesting observation from the percentages outlined is that although HPV 16 is the most prevalent genotype in cervical and head and neck cancers, it contributes to a greater proportion of head and neck than cervical SCCs, from the research conducted to date on high-risk HPV subtypes [134]. The converse is that HPV 18 appears to have higher oncogenic potential in the cervix in contrast

to the head and neck region [134]. No identifiable anatomical, physiological, or biochemical features have been elucidated as to why this is the case.

1.2. Differences between HPV Positive and Negative Squamous Cell Carcinoma of the Head and Neck Region

1.2.1 Genomic Characterisation of HPV positive versus HPV negative Squamous Cell Carcinoma of the Head and Neck Region

Research from the 1990s has provided instrumental knowledge regarding the carcinogenesis of HNSCC [70]. The presence of mutations in *TP53, CDKN2A*, *PIK3CA* and Phosphatase and tensin homolog (*PTEN*), which are involved in the receptor tyrosine kinase/RAS/Phosphoinositide 3 kinase (RTK/RAS/PI3K) pathway; Notch homolog 1, translocation-associated (Drosophila) (*NOTCH1*), which is linked with squamous differentiation; *F-Box* And WD Repeat Domain Containing 7 (*FBXW7*) tumour suppressor gene, which has a downstream effect on *NOTCH1*, were the initial mutations detected in HNSCC cases [132, 135-138].

A paper published in 2000 by Gillison *et al.* established that HPV positive OPSCCs have a distinctive mutational landscape compared with HPV negative counterparts [139]. This study was the first to highlight the inverse relationship between *TP53* mutation and HPV positivity [139].

In 2011, two studies utilised whole genome sequencing to analyse the genetic framework of SCCs of the head and neck region [140, 141]. These studies conducted by Stransky *et al*. and Agrawal *et al*. investigated not only OPSCC, but

SCCs from other sites including the hypopharynx and oral cavity. Both studies documented that HPV positive SCCs from the head and neck lacked mutations in the *TP53* gene [140, 141].

In 2015 the Cancer Genome Atlas (TCGA) published its work underpinning the landscape of genomic alterations associated with HNSCC [142]. In total, 279 HNSCC cases were analysed in relation to DNA and RNA structural alterations, and somatic mutations, of which 36 cases of HPV positive HNSCC were identified within the cohort. Most of the HPV positive cases arose from within the oropharynx, with the minority from within the oral cavity. A comprehensive list of mutations observed in HNSCC based on their HPV status from published literature to date is illustrated in *Table 1.2* and *Figure 1.7.* The cited literature focuses on HPV 16 and 18 positive HNSCC with figures derived from the oropharynx predominantly, with occasional references to other sites within the head and neck where HPV positivity was detected.

HPV Status	Gene	Percentage of Cases
Positive	*HPV E6/E7*	**100**
	PIK3CA	**10 - 56**
	TRAF3	**5 - 28**
	TP63	**11 - 28**
	E2F1	**19**
	Let 7c	**17**
	NOTCH	**8 - 17**
	PTEN	**6 - 15**
	KMT2D	**13 - 14**
	EP300	**10 - 12**
	ZNF750	**11**
	CYLD	**10**
	RB1	**10**
	HRAS, KRAS	**10**
Negative	*TP53*	**83 - 100**
	CDKN2A	**25 – 58**
	CCND1	**22 - 55**
	Let-7c	**40**
	PIK3CA	**13 - 34**
	FAT1	**14 - 32**
	FADD	**32**
	TP63	**28**
	NOTCH1	**13 - 26**
	SYNE1	**22**
	MUC16	**19**
	USH2A	**18**
	ZFHX4	**14 - 16**

Table 1.2 Continued		
	MLL2	**16**
	EGFR	**12 - 15**
	MYC	**14**
	LRP1B	**14**
	NFE2L2	**14**
	PIK3CB	**13**
	URB5	**13**
	PTEN	**12**
	CASP8	**11**
	FGFR1	**10**

Table 1.2. *Table illustrating the most frequent mutations identified in HPV positive and negative head and neck squamous cell carcinoma compiled from data from sources. Results are compiled from cited work [137, 140-154].*

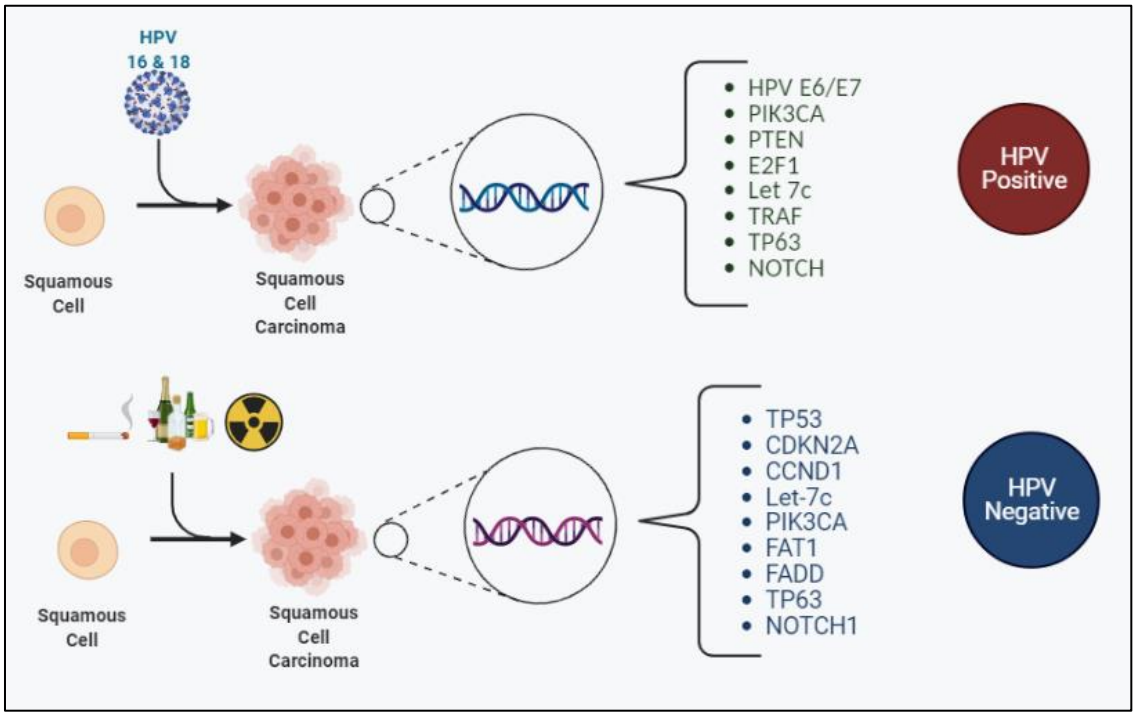

Figure 1.7 *The most frequently encountered somatic mutations for HPV negative and HPV positive Squamous cell carcinomas of the head and neck region, most predominantly within the oropharynx. Figure complied from cited resources [137, 140-154].*

Source: Brennan et al. [66]

The most frequently overlapping mutated genes amongst HPV positive and negative OPSCC were *TP53, CDKN2A, FAT1, PIK3CA*, Lysine Methyltransferase 2D (*KMT2D*) and *NOTCH1*. These play a key role in the RTK/RAS/PI3K pathway as illustrated in *Figure 1.8 [144]*. Other frequent mutation genes within OPSCC include *let-7c* and *FGFR3*, which are also linked to the RTK/RAS/PI3K pathway [144, 155].

Phosphoinositide 3 kinases are a family of enzymes subdivided into three classes. They are activated by receptor tyrosine kinases (RTKs) and g-protein coupled receptors (GPCRs). The class I subgroup of the PI3K family is most frequently

associated with mutational dysregulation in HPV positive OPSCCs. Activation leads to phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3, 4,5-triphosphate (PIP3), which subsequently activates protein kinase B (AKT). AKT activates further targets downstream including mammalian target of rapamycin (mTOR). mTOR is divided into two complexes: Complex 1 and 2, which control biochemical and metabolic functions of the cell including lipid, protein and carbohydrate metabolism [156]. The accumulative effect of this deregulated PI3K/AKT/mTOR pathway results in cell growth, differentiation, survival and migration [157, 158]. Furthermore, *PTEN* is frequently mutated in head and neck cancers, and its loss provides further amplification of the PI3K pathway [158].

In addition, mutations in *FGFR3* permits dysregulated signal transduction and subsequently activates mitogen-activated protein kinase (MAPK), PI3K/AKT pathways [159].

The PI3K/PTEN/AKT/mTOR pathway has been identified as a key component of carcinogenesis in the HPV positive cohort to date, as illustrated in *Figure 1.8*. With this knowledge, mTOR inhibitors are being trialled in patients with advanced/metastatic disease irrespective of HPV status [156]. The most notable of which is the TEAMHEAD Phase II trial, which showed disease stabilisation in 57.6% of cases and tumour shrinkage in 39.4% of cases treated with Temsirolimus, in recurrent/metastatic HNSCC post treatment failure [160]. Other trials are ongoing at present.

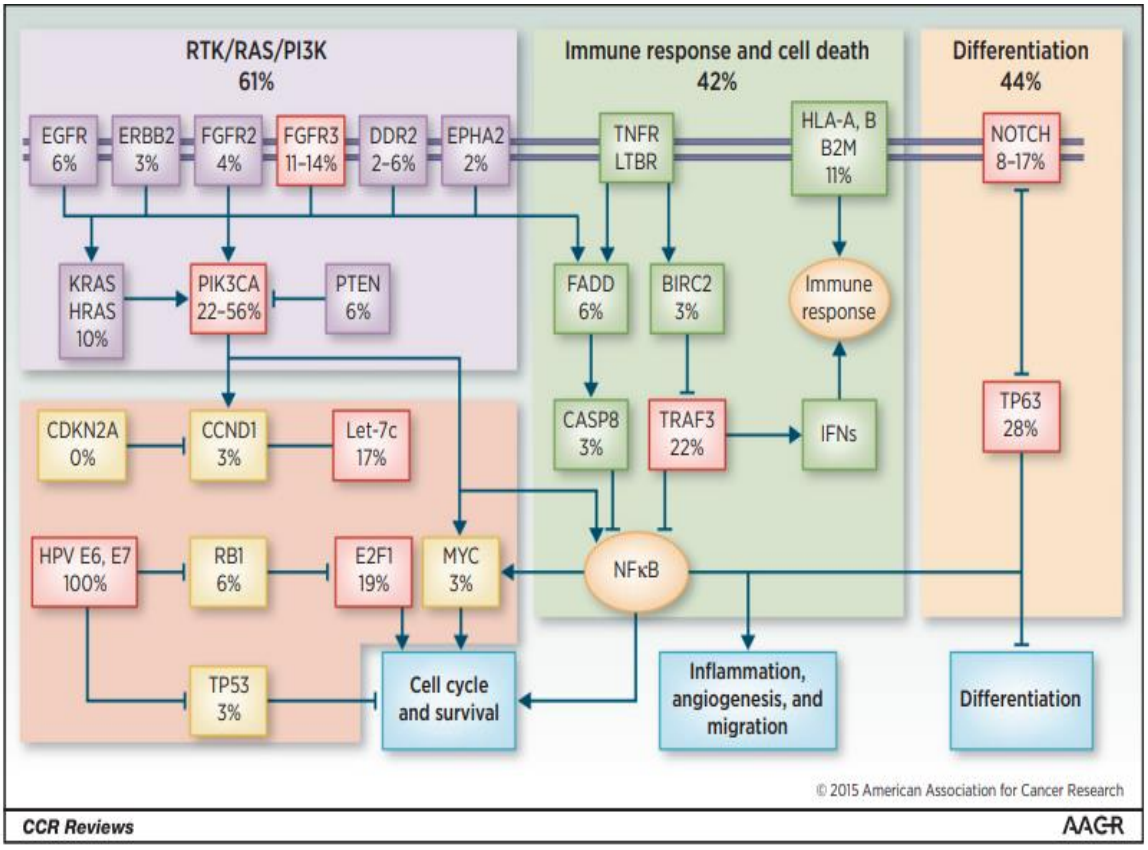

Figure 1.8. *Signalling pathways affected in HPV positive head and neck cancers, with respective end point implications to cell function.*

Source: Rusan et al. [144]

1.2.2 Prognosis for patients with HPV Positive OPSCC

HPV positive OPSCC are associated with young white males; a male to female ratio of 3:1; classically non-smokers, and it has been deemed to be a distinct clinical entity from HPV negative disease [44, 45]. Although HPV is a risk factor for the development of OPSCC, the overall prognostics are favourable in contrast to those who are negative [161, 162]. From the literature published during the advent of derogation into two cohorts based on HPV status, HPV positive OPSCC showed a 60% reduction in risk of death from cancer, taking into account age, alcohol consumption and lymph node involvement [139].

Subsequent studies have demonstrated increased sensitivity to treatment, with higher response rates to radiation therapy, induction chemotherapy and chemo-radiation therapy (CRT) [44, 163]. The two-year progression free survival (PFS) rates for HPV positive and negative HNSCC range from 72% to 86%, and 50% to 75%, respectively [44]. The two-year overall survival (OS) rates range from 88% to 94%, and 58 % to 77%, respectively [44]. The five-year survival from studies in Canada and Austria show a greater divergence with figures of 83 % and 85.7 %, compared to 11.1 % and 22.2 % (HPV positive *vs*. negative) [164, 165].

The following are some of the factors believed to contribute to the differences in outcome:

1. Given the characteristic younger age at diagnosis, individuals may have fewer co-morbidities, with a good baseline performance status prior to starting treatment [10, 162].

2. Studies have shown that HPV OPSCC has higher radio-sensitivity. Mechanisms by which this has been illustrated include the role of *TP53* and high oxygenation levels within HPV positive tumours in contrast to HPV negative tumours [41].

   HPV negative OPSCCs are associated with mutated *TP53* gene, which results in deregulation of the cell cycle [166]. However, HPV positive OPSCC are shown to express wild type p53 protein, thus maintaining a degree of normal functionality. RT induces cellular stress and damage through the activation of p53 responsive pro-apoptotic genes such as BCL2 Associated X, Apoptosis Regulator (*Bax*) [41]. This is believed to play a key

role in the favourable response rates of HPV positive OPSCC to RT [10, 41, 167].

A further theory is related to the fact that HPV positive OPSCC are less hypoxic than HPV negative OPSCC. Oxygen is required for free radical formation, and thus the effectiveness of RT. Higher oxygen and oxygen free radicals levels derived from HPV may thus be linked to overall improved radio-sensitivity [41].

3. Another proposed mechanism is via immune regulation. Orthotopic models have shown immunocompetent mice do better than immunosuppressed mice with HPV positive head and neck cancers [168-170]. HPV OPSCC patients have a shift towards a greater number of tumour infiltrating lymphocytes such as CD8 positive T-Cells, CD8 positive T-cells who express PD1, and C4 positive T-cells [168-170]. Studies have also shown that patients with HPV positive tumours have a greater number of effector memory T-cells in peripheral blood [168]. The proposition is thus that immune response to tumour cells harbouring HPV may provide a significant anti-tumour effect.

4. Finally, differences in the mutational status of HPV positive *vs*. HPV negative OPSCC are believed to benefit the HPV positive cohort, with the listed mutations illustrated in *Table 1.2*. Of note however, there is an overlap with certain mutations in both cohorts. Thus, further robust analysis into a link between specific genetic mutations and survival are warranted.

1.2.3 Treatment guidelines for HPV Positive OPSCC

Despite varied outcomes, present treatment guidelines for HPV positive disease are per those for HPV negative [151, 171]. In keeping with the National Comprehensive Cancer Network (NCCN) clinical practice guidelines in Oncology for Head and Neck cancers and the European Head and Neck Society, the European Society for Medical Oncology, and the European Society for Radiotherapy and Oncology (EHNS-ESMO-ESTRO) clinical practice guidelines, HPV detection should not change treatment plans [86, 171, 172]. The standard therapy for newly diagnosed HNSCC is dependent on the TNM stage and ranges from RT, CRT and/or surgical intervention [171].

There are numerous factors currently being studied to address alternate strategies for HPV positive OPSCC.

1.2.3.1 De-Intensifying Therapies

Curative therapies, although effective in their aim to improve PFS and OS, are not without their risks and complications. The toxicity associated with chemotherapeutics and RT can be extremely challenging and debilitating, thus it is important to provide an adequate dosage of a therapeutic agent without inducing localised and/or widespread complications. This is extremely important in younger patients with HPV positive disease, who will potentially live longer, but could have poorer quality of life with side effects including xerostomia, dysphagia and the requirement for parenteral feeding tubes [173]. This has resulted in clinical trials examining de-intensifying therapies for HPV positive OPSCC to reduce treatment induced morbidity and improve quality of life. Alternative treatment strategies

investigated include dose reduction or elimination of chemotherapy, implementing alternative systemic therapies, dose reduction and field reduction of RT, and/or alternative surgical techniques [174].

Intensity modulated RT involves administration of a reduced treatment dose, with studies administering fractions ranging from 30 to 60 Gy [175-178]. Preliminary results from phase II and III trials show no inferiority in maintaining loco-regional control and PFS. However, the follow up time frame is limited, with no long-term follow-up studies published [176-178]. A further strategy is that of targeted reduced dosage of RT, with research ongoing in Memorial Sloan Kettering regarding delivery of a standard 70 Gy to visible disease, with delivery of 30 Gy to elective regions (sites with no gross identifiable disease) [175]. Of note, there is no provision within the EHNS-ESMO-ESTRO guidelines for amendments to the set protocol guidelines for radiation dose reduction for HPV positive OPSCC given the prematurity of this data [171].

1.2.3.2 Alternative Chemotherapeutic Agents

As highlighted above, mechanisms to reduce toxicity and subsequently improve quality of life relate to administration of an alternative agent to the mainstay systemic chemotherapeutic agents. Such agents utilised include Cisplatin, or a platinum therapy combined with 5-Fluorouracil. One of the proposed substitutes involves targeting receptors linked to pro-oncogenic activity in conjunction or as an alternative to systemic chemotherapy. Molecular analysis of OPSCC identified high levels of epidermal growth factor receptor (EGFR) expression, and as illustrated in *Table 1.1*, such mutations are more predominant in the HPV negative cohort [179, 180]. This high expression has been linked to reduced OS, reduced radio-

sensitivity, and increased recurrence post RT [179, 181, 182]. EGFR, a transmembrane receptor, is a member of the erbB category of type I RTKs with downstream signalling involving the microtubule associated protein kinase/eukaryotic protein kinase (MAPK/EPK), Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) and PI3K/AKT pathways. These pathways impact cell proliferation, cell survival, evasion of apoptosis, angiogenesis, and cell migration [182, 183]. The dysregulation of EGFR within this cohort of patients has resulted in the utilization of Cetuximab, an Immunoglobulin G1 (IgG1) targeted monoclonal antibody, that targets EGFR specifically.

EGFR targeted therapies for HNSCC have shown improvement in OS benefit when administered with RT, with a landmark study showing such in 2000 by Bonner *et al.* [179, 184]. In this study, patients were included irrespective of EGFR status. The findings of this study prompted a new focus of research investigating Cetuximab in HPV positive OPSCC, given the greater duration of survival for younger patients who would subsequently have to live with higher levels of morbidity secondary to conventional treatment. The De-ESCAlaTE trial replaced Cisplatin with Cetuximab with concomitant RT for patients with HPV positive OPSCC. This study showed no benefit in reducing toxicity, however, a reduction in tumour control was observed [185, 186]. A further study by Gillison *et al.* showed that RT plus Cetuximab demonstrated inferior OS and PFS compared to RT plus cisplatin, with similar toxicity rates [186, 187]. In summary, Cetuximab has not been shown as an alternative for systemic chemotherapy and the current treatment guidelines issued do not endorse the usage in replacement of systemic chemotherapy in treating HPV positive OPSCC [171]. However, it is important to

reiterate that studies focusing on the utilisation of cetuximab have not stratified patients based on their EGFR or HPV tumour status.

1.2.3.3 Radiation Therapy

Further de-intensification measures such as replacing CRT with RT alone have been trialled. A retrospective study from 2013 showed a comparable outcome for HPV positive OPSCC treated with RT alone in contrast to those treated with CRT [174, 188]. However, preliminary results from a phase II trial reviewing intensity modulated RT with chemotherapy versus modulated RT alone, showed a 2 year PFS of 90.5% for combined therapy vs. 87.6% for RT alone [176]. Additional studies are ongoing which are reviewing various modalities to reduce intensity and thus toxicity of therapy without compromising cancer control. One such Phase II trial examines administration of induction Cisplatin chemotherapy, followed by cetuximab with reduced dose RT for HPV-positive resectable stage III/IV OPSCC [189]. The study showed reduced and delayed swallowing issues for those who received induction chemotherapy and reduced dose RT and recommended further large-scale studies. A Phase III trial, the Quarterback trial, of HPV positive OPSCC examined a reduced dose CRT after induction chemotherapy versus standard CRT. The result showed similar PFS and OS in both groups [190].

1.2.3.4 Organ Preservation Surgery

The utilisation of ‘organ preservation surgery’ to ensure minimal long term morbidity is an increasing area of interest acknowledged within current guidelines [171]. Results from a Phase II trial in 2020 utilising transoral robotic surgery with

intensity modulated RT has shown promising results in HPV positive OPSCC [191]. Additional trials reviewing less invasive surgical techniques include PATHOS (primary surgical de-escalation trial in Post-Operative Adjuvant Treatment of HPV-Positive Tumours) and ADEPT (Adjuvant De-escalation, Extracapsular Spread, P16 Positive, Transoral trial) are ongoing [192, 193]. The overall aim of such techniques in conducting a localised surgical resection without deformative surgical intervention in addition to chemotherapy and radiotherapy is to achieve cancer control, minimise morbidity and improve overall survival rates.

In conclusion, the treatment for HPV positive OPSCC has not differed from its HPV negative counterpart, despite over a decade of investigations and research. The targeted focus of reduced RT dosing, the utilisation of immunomodulatory agents and transoral robotic surgery appear to be the mainstay of the research focus at present. Although currently reserved for recurrent unresectable/metastatic disease, the prescribing of immune checkpoint inhibitors may be an area worth investigating in this cohort of patients to limit morbidity from non-targeted toxic therapeutic agents.

1.3 HPV Positive OPSCC Linked with Locoregional Recurrence and/or Disease Progression

1.3.1 HPV Genotypes Linked with Locoregional Recurrence and/or Disease Progression

Despite the definitive association of HPV positivity with better overall outcomes in OPSCC, there is limited research to date studying the impact of each HPV genotype

with respect to OS and PFS. This may be in part due to the vast majority of HPV positive OPSCC being positive for the HPV 16 genotype (*Figure 1.6)*. Lei *et al.* highlighted that HPV 18 is associated with a worse prognosis than HPV 16 in invasive cervical carcinoma [194]. A review of nearly 700 cases of HPV positive invasive cervical cancer from an American cancer registry showed an improved 5 year survival in HPV 31/33/44/52/58 subtypes in contrast to HPV 16 and HPV 18 [195].

Regarding the head and neck region, a multicentre study in Korea published in 2015 revealed that tonsillar carcinomas which were positive for the HPV 18 genotype were associated with shorter disease-free interval (DFI) than that of the HPV-negative and HPV 16 positive groups [196]. These findings were further supported by Bratman *et al.* and TCGA illustrating that HPV positive OPSCC subtypes other than HPV 16 had a more favourable OS [137, 142, 197, 198]. However, such results are conflicting, with a recent study by Ziai *et al.* which suggests that HPV 16 confers a worse OS and PFS trend [198]. It is worth noting that such findings were not statistically significant. Thus, correlating particular genotypes with PFS or OS may be an area worth exploring in the future.

1.3.2 Genetic Aberrations Linked with Locoregional Recurrence and/or Distant Metastasis

To date there is limited data examining the genetic aberrations associated with HPV positive OPSCC who develop locoregional (LR) and/or distant metastasis (DM) [147, 199-201]. Two key studies by Reder *et al.* and Harbison *et al*. collectively analysed 28 patients with documented recurrences [147, 199]. The working

hypothesis was that HPV positive OPSCC that recur share a mutational landscape similar/in keeping with HPV negative OSPCC, thus explaining the poor prognosis [147, 199].

Combining the two studies in HPV positive OPSCC, the most common mutations identified amongst the HPV positive OPSCC groups included, *TP53, RB1, KMT2D, PIK3CA, TAF1, CYLD, EP300, PEG3, STAT3, BCL6, FBXW7, NCOR1, NSD1, DDX3X, PDGFRA, NOTCH1* [147, 199]. Mutations in *TSC2, BRIP1, NBN, TACC3, NFE2L2, STK11, HRAS, PIK3R1, TP63 and FAT1* were identified in the recurrence group only (*Table 1.3*). *FGFR3, B2M, TRAF3, SMAD2, ARID, MAPK1 and MAPK2K2* mutations were observed in those who did not recur [147, 199].

Mutations Detected in Recurrence Group of HPV Positive OPSCC
TSC2
BRIP1
NBN
TACC3
NGE2L2
STK11
HRAS
PIK3R1
TP63
FAT 1

Table 1.3 *Most frequent mutations identified in patients with HPV positive OPSCC who developed recurrence. [147, 199]*

Regarding the mutations identified solely in the recurrence group, *PIK3R1, TSC2 and STK11 (LKB1)*, these are involved in the PI3K/AKT/mTOR pathway. Tuberous Sclerosis Complex subunit 2 (TSC2) is a tumour suppressor gene which produces the protein tuberin. This protein interacts with the hamartin protein produced by Tuberous Sclerosis Complex subunit 1 (*TSC1*) [202]. Hamartin-tuberin complex exhibit a GTPase activating activity on RHEB (Ras homolog enriched in brain). The downstream effect is the inhibition of mTOR, which impacts the PI3K/AKT/mTOR pathway [202, 203]. Mutated TSC2 results in upregulation of this pathway. The tumour suppressor gene serine threonine kinase 11 (*STK11*), also referred to as liver kinase B1 (*LKB1*), activates adenosine monophosphate-activated protein kinase (AMPK), which has an inhibitory effect on mTOR. Mutation of *STK11* leads to the upregulation of the mTOR pathway [204, 205].

TP63 is a tumour suppressor gene from the same family as *TP53*. The *TP63* gene is expressed in numerous isoforms, most relevant thus far to tumorigenesis are TAp63 and ΔNp63 [206]. *TP63* has been linked to cell survival, senescence, cellular differentiation and apoptosis [206]. The loss of function of the *TP63* gene, can result in the activation of MAPK. This can promote the upregulation of the Mitogen Associated Protein Kinase - Signal Transducer And Activator Of Transcription 3-Matrix Metalloproteinase 5 axis (MAPK- STAT3- MMP), which has been shown to promote metastasis [207]. It is worth noting that although this mutation was solely identified in the recurrence group of HPV positive OPSCC from Reder *et al.* and Harbison *et al.*, such mutations have also been identified in the HPV negative cohort in other studies [147, 199].

The next gene mutation linked with HPV positive OPSCC who recur is the *BRIP1* (BRCA1 interacting protein C-Terminal Helicase 1). This gene is associated with

Fanconi's anaemia, a condition linked to risk of HNSCC [208]. *NBN* gene (also known by NBS1, Nijmegen Breakage Syndrome) produces protein nibrin, which plays an instrumental role in DNA repair [208-210].

A further mutation in the transforming acidic coiled coil containing protein 3 (*TACC3*) gene has been discovered [147, 199]. This gene produces a non-motor protein that functions to promote microtubule growth from the centrosome, thus providing stability to the mitotic spindle. This occurs via a transforming acidic coiled-coil protein 3 (*TACC3*)/colonic, hepatic tumour overexpressed gene (ch-TOG)/clathrin complex [211, 212].

Additional mutations have been found in nuclear factor–erythroid-2–related factor-2 (*NFE2L2*), a gene that encodes the transcription factor NF-E2-Related Factor 2 (Nrf2). Nrf2 plays a role in the antioxidant response within the cell, with mutation leading to cellular resistance to antioxidant damage, thus ensuring cell survival [213, 214]. FAT1 gene is a member of the cadherin family. It is a homologue of Drosophila tumour suppressor gene fat, and is involved in cell movement and inhibition of cell growth [215].

Harvey rat sarcoma viral oncogene homolog is a member of the RAS family of oncogenes, which is frequently mutated in a variety of cancers. The family is subdivided into *HRAS, KRAS* and *NRAS*. The *RAS* family of oncogenes are extremely important in tumorigenesis owing to their key role in signal transduction pathways. The RAS protein alternates between being bound to a GTP or GDP, with GDP being the inactive state. RAS signalling plays a key function in cellular proliferation, survival, angiogenesis and differentiation [216].

There have been studies conducted to elicit whether particular somatic mutations are associated with recurrent and/or metastatic disease, which could serve as a prognostic marker.

NOTCH1 and *PTEN* mutations were linked with a reduced recurrence free survival in patients with HPV positive OPSCC [217], with *PIK3CA* mutations linked with reduced disease free survival (DFS) in those on de-intensified CRT [218]. Conversely, *FGFR3* mutations in HPV positive OPSCC are linked with improved DFS [219]. A further study highlighted that *FGFR3* mutations were predominantly identified in an HPV negative OPSCC cohort, and in combination with mutant *TP53* was linked with a worse overall prognosis [220]. Thus, extrapolating these data, combined mutant *TP53* and mutated *FGFR3* in the HPV positive OPSCC cohort is linked to improved OS.

There are two principal points regarding genetic aberrations in OPSCC tumours associated with LR and/or DM. Firstly, although hypothesised that the group with LR and/or DM have genetic mutations in line with the HPV negative cohort, Harbison *et al.* concluded that HPV related OPSCC that did or did not recur had more genomic features in common with each other than with HPV-unrelated tumours [147]. Secondly, the caveat from the literature is that the results depicted are all derived from studies with a small cohort of patients, thus caution is warranted regarding interpretation. However, this has set the stage for ongoing innovative research in this area to assess what/if any mutational features may thus be associated with recurrence, which may provide in-depth future knowledge for targeted therapies.

1.3.3 Treatment guidelines for patients with HPV Positive HNSCC with Recurrence and/or Metastasis

In patients with LR and/or DM, the mainstay of treatment is dependent on numerous factors including site of recurrence, resectability and prior treatment [171]. Treatment regimens can vary from surgical intervention, RT, systemic chemotherapy and utilization of novel agents including immune checkpoint inhibitors (ICIs) [171, 174].

As alluded to previously, targeting immune checkpoints can help to enhance the immune response to the cancer. The main targets include cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and PD1/PD-L1 [221].

Lyford-Park *et al.* in 2013 published a seminal paper demonstrating the role of high PD1/PD-L1 expression in providing an environment for HPV infection and persistence, with provision to eliminate the immune regulation and resultant tumorigenesis [54]. Studies have illustrated a strong correlation between HPV positivity and PD1/PD-L1 expression, when PD-L1 cut offs are assessed at varying scores, including examination of membranous and/or cytoplasmic staining [222-225]. PD-L1 expression has been linked to improved outcomes in certain studies addressing HNSCC [222, 223, 226]. However a recently published systemic review and meta-analysis, showed PD-L1 expression was linked with improved OS, with HPV status showing no impact on the findings [226].

Nivolumab, an IgG4 anti–PD1 monoclonal antibody, has been trialled for recurrent HNSCC, irrespective of HPV status, and has shown improved OS compared to those on single agent Docetaxel, Methotrexate or Cetuximab [227]. Although this study looked at all head and neck cancers, the HPV positive cases had longer

duration of OS [228]. Thus, this may be an area for clinical studies in the future. The KEYNOTE-048 study proved that the combination of standard chemotherapy with Pembrolizumab significantly improved OS than chemotherapy alone for recurrent or metastatic disease [229]. However, this study did not consider the HPV status.

A further phase II trial, HAWK, examined the utilization of Durvalumab monotherapy versus systemic chemotherapy in patients with recurrent or metastatic HNSCC that had high PD-L1 expression in tumour cells. When stratified based on site and HPV status, patients with HPV-positive OPSCC had a response rate of 30 %, in contrast to 10.8 % in the HPV negative cohort [230, 231].

Recently, the Food and Drug Administration (FDA) approved the utilisation of Pembrolizumab in combination with chemotherapy (platinum-based therapy plus 5-fluorouracil) as first line treatment for metastatic/recurrent unresectable HNSCC irrespective of PD-L1 combined positive score (CPS), and as a sole line of treatment if the PD- L1 CPS is ≥ 1, which is assessed via IHC [171]. However, the European Medicines Agency (EMA) guidelines differ; Pembrolizumab administration as first line treatment for metastatic/recurrent with or without chemotherapy (platinum-based therapy plus 5-fluorouracil) only when the PDL-1 CPS is ≥ 1 [171]. Neither guideline delineates treatment based on HPV status.

In summary, treatment of recurrent and/or metastatic disease is dependent on numerous factors and treatment regimens are varied. However, the recent approval by the FDA and EMA for Pembrolizumab indicate that ICIs are now playing a pivotal role in treatment of metastatic/recurrent unresectable HNSCC. Once again, there are no treatment regimens based on HPV tumour status at present.

1.4 Study Aims and Objectives

HPV driven cancers within the oropharynx have marked pathogenic differences in contrast to HPV driven cervical cancers. HPV proffers a favourable prognosis for HPV positive OPSCC in contrast to its HPV negative counterparts. However, cases of recurrence of HPV positive OPSCC do occur and are linked with poorer outcomes. There is limited research to date examining the genotypic strains and the mutational landscape of HPV positive OPSCC patients who experience LR and/or DM. With ongoing research into de-intensifying therapeutic regimens for HPV positive OPSCC, the identification of those individuals likely to develop LR and/or DM is paramount. Concentrating on genotypic and/or mutational patterns in the recurrence group, as well as identification of individuals with residual virus within the oropharynx post cancer treatment, may result in improved stratification of treatment for patients with HPV positive OPSCC.

The aims of this study are as follows:

1. Determine the prevalence of various HPV DNA genotypes in HPV positive OPSCC from archival tumour tissue.
2. Establish if there is a correlation between specific HPV DNA genotypes and those who develop LR and/or DM.
3. Determine the prevalence of specific somatic mutations including BRAF, EGFR, ERBB2, KIT, KRAS, MET, NRAS, PDGFRA in HPV positive OPSCC from archival tumour tissue.
4. Assess if there is a correlation between specified mutations outlined and those who develop LR and/or DM.

5. Establish if there is a correlation between patient demographics including age, gender, smoking status, alcohol consumption, tumour site, tumour clinical stage, treatment regimen received for primary tumour, DFI, with the identified HPV genotype and specified somatic mutations.
6. Establish if there is a correlation between patient demographics including age, gender, smoking status, alcohol consumption, tumour site, tumour clinical stage, and treatment regimen received for primary tumour, DFI, with the development of LR and/or DM
7. Establish the feasibility of molecular interrogation of drivers of disease progression on archival FFPE tumour tissue in HNSCC.

Chapter 2

Materials and Methods

2.1 Patient Cohort

Inclusion Criteria:

Patients diagnosed with HPV positive OPSCC [ICD10 C01, C02.4, C05.1, C05.2, C09.0-C09.9, C10.0-C10.9, C14.2)] between 01/01/2007 and 31/12/2016 detailed within the St. James's Hospital (SJH) Head and Neck cancer database, which encompasses cases from SJH, Tallaght University Hospital (TUH) and the Royal Victoria Eye and Ear Hospital (RVEEH), were deemed eligible for inclusion in this study. All participants were over the age of 18 years. Each patient required a FFPE tumour tissue either from a biopsy and/or resection specimen archived in the Histopathology Department in SJH, TUH or RVEEH. Each patient required a minimum confirmatory p16 IHC on their tumour biopsy/resection specimen.

Exclusion Criteria:

Participants with OPSCC [ICD10 C01, C02.4, C05.1, C05.2, C09.0-C09.9, C10.0-C10.9, C14.2)], from the SJH Head and Neck cancer database (01/01/2007 - 31/12/2016) whose tumour was negative for HPV via immunohistochemistry were excluded. Patients under the age of 18 years were also excluded.

Patients still alive and eligible (as of 01/01/2020) were contacted by post (information pack included a Cover Letter, Patient Information Leaflet and Consent Form; *Appendix 1*) and asked for their written consent if they wished to take part in this research study. Consent provided access to their FFPE tumour blocks, hospital chart, Electronic Patient Records and histopathology reports on the Laboratory Information System. The cut-off date for returning of valid consent forms was the 30/06/2020.

Ethical approval from the SJH/TUH Joint Research Ethics Committee and RVEEH Ethics Committee was provided to access patients who were eligible but deceased.

Sixty Seven patients with HPV Positive OPSCC identified within the SJH Head and Neck Cancer database met the inclusion criteria.

The patient charts in SJH, Electronic Patient Record in SJH, histopathology reports within the Laboratory Information System hospital chart in SJH, TUH and RVEEH were examined with all pertinent data to the study entered into an excel document. The cut-off date for data analysed relating to patient follow up was the 30/06/2022.

Definitions:

Locoregional Recurrence: Recurrence of SCC within the region of the primary tumour within head and neck and/or neck nodal involvement post treatment.

Distant Metastasis: Dissemination of the primary SCC to a site outside of the head and neck region post treatment.

Disease Free Interval: Time frame between cessation of treatment for primary tumour and the development of locoregional recurrence and/or distant metastasis.

Sufficient Tumour Tissue: Each H&E slide was reviewed by light microscopy to ensure the presence of at minimum 100 tumour cells.

2.2 DNA Extraction

2.2.1 Formalin Fixed Paraffin Embedded Selection

A H&E slide and p16 IHC slide from the relevant hospital site for each case was examined by light microscopy to ensure the presence of a minimum 100 SCC tumour cells. For cases in which there were numerous FFPE blocks and corresponding H&E slides, the slide/block with the most cancer cells evident under light microscopy was chosen.

2.2.2 Sectioning of FFPE

FFPE blocks were cut using a microtome at measurements between 5 to 10 µm, with a total of 5 sections per case. To ensure no cross-contamination between samples numerous measures were put in place:

(i). Non-latex gloves were utilised at the time of cutting, with a change of gloves between each case

(ii). The microtome blade was changed between each case

(iii). The microtome was cleaned with 10 % bleach, water, and molecular grade Ethanol between each case

(iv). Ten separate 10 µm sections were taken from a blank FFPE block between every case. The purpose was to minimise the risk of residual tissue from prior sample that may have remained on any component of the microtome after cleaning.

(v). Forceps were cleaned with bleach, water, and molecular grade Ethanol between each case

(vi). FFPE sections were placed directly into cooled labelled micro-centrifuge tubes

2.2.3 DNA Purification from FFPE Tissue Sections

DNA purification from FFPE tissue sections was conducted utilising the QIAamp® DNA FFPE Kit (Qiagen) according to manufacturer's instructions.

Briefly, 1 mL Xylene was added to the FFPE tissue sections in a 1.5 mL micro-centrifuge tube and vortexed for 10 sec. The sample was then centrifuged for 2 min at room temperature. Once completed, the supernatant was removed by pipetting. One mL molecular grade ethanol was then added, and the sample vortexed and centrifuged as above. The supernatant was removed, and the micro-centrifuge tube was left with the lid open in the fume hood at room temperature for 10 min.

Following this, 180 µL Buffer ATL and 20 µL Proteinase K was added to each sample. The sample was vortexed, and then incubated for 1 h at 56 ℃, followed by 1 h at 90 ℃. The specimen was centrifuged briefly, following which 200 µL Buffer AL was added and the sample vortexed. Two hundred µL molecular grade Ethanol was then added to the sample, with the sample then vortexed and centrifuged.

The lysate was then transferred to a QIAamp MinElute column, which was placed in a 2 mL collection tube and centrifuged for 1 min at 8,000 rpm. The residual fluid was removed and the QIAamp MinElute column was placed into a clean collection tube.

Five hundred µL Buffer AW2 was added and centrifuged for 1 min at 8,000 rpm. The residual fluid was removed and the QIAamp MinElute column was placed into

a clean collection tube. The QIAamp MinElute column and new collection tube were placed in the centrifuge once more for 3 min at 14,000 rpm.

The QIAamp MinElute column was then placed in a clean 1.5 mL microcentrifuge tube. Fifty µL Buffer ATE was added to the centre of the QIAamp MinElute column membrane. The tube was incubated at room temperature for 1 min and then placed into the centrifuge for 1 min at 14,000 rpm, following which the samples was stored at in a - 20°C freezer.

2.2.4 DNA Quantification

The purified DNA was quantified using a Qubit® Fluorometer, Qubit dsDNA HS Assay Kit (Invitrogen by Thermo Fisher Scientific) and Qubit specific micro-centrifuge tubes according to manufacturer's instructions.

Briefly, the Quantification working solution was made by adding 1 x n µL (where n = number of standards plus number of samples) Quant-iT reagent to Quant-iT™ Buffer 199 x n µL. This solution was denoted the Quant-iT™ working solution. One hundred and ninety µL Quant-iT™ working solution was added to 10 µL Standard 1, and 190 µL Quant-iT™ working solution was added to 10 µL Standard 2. One hundred and ninety eight µL Quant-iT™ working solution was added to 2 µL from each DNA sample obtained outlined in section 2.2.3. The sample was vortexed for 2-3 sec and incubated at room temperature for 2 min. The specimen was then inserted in the Qubit™ fluorometer with reading provided with concentrations in ng/mL.

2.3 DNA Library Preparation

2.3.1 Ion AmpliSeq Design Panel

Utilising the Thermo Fisher Scientific Ion AmpliSeq Designer (Ion Torrent™ by Thermo Fisher Scientific), a specified panel was designed by Associate Professor Magnus Stougaard, Institute of Pathology, Aarhus University Hospital, Denmark for HPV genotyping [232]. The panel composed of 63 amplicons, covering an amplicon range of 125-175 base pairs (bp), with a panel size of 1.68 kilobytes (kb). Gender markers were also utilised, Ion AmpliSeq™ Sample ID Panel (Thermo Fisher Scientific). The full panel of HPV hotspots targeted is outlined in *Appendix 2*. The list of HPV genotypes targeted is illustrated in *Table 2.1*.

HPV Genotypes		
High Risk	Probable High Risk	Low Risk
16	26	6
18	53	11
31	66	
33	67	
35	70	
39	73	
45	83	
51	30	
52	34	
56	69	
58	85	
59	97	
68		

Table 2.1 *List of the HPV Genotypes Targeted via Ion Torrent Next Generation Sequencing.*

For each of the 27 HPV types included in the NGS panel, eight amplicons were designed. Two amplicons were designed for each of the E6 and E7 oncogenes, one amplicon for L1 and three amplicons were designed for E2 [233].

In order to ensure normalisation and standardisation for this study, four human reference genes, or 'housekeeping' genes were added to the original panel devised. They include Basic Transcription Factor 3 (BTF3), Ribosomal Protein S27a

(RPS27A), Ribosomal Protein L32 (RPL32) and Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1 (UBA52).

2.3.2 DNA Target Amplification

The DNA target amplification library was prepared utilising the Ion Ampliseq™ Library Kit Plus User Guide (Ion Torrent™ by Thermo Fisher Scientific) and completed according to manufacturer's instructions, as briefly described below. The 2X Single Primer Panel Pool master mix components were thawed on ice and the master mix was prepared on ice, as per *Table 2.2*.

Component	Volume/Reaction
5X Ion Ampliseq™ HiFi Mix	4 µL
2X Ion Ampliseq™ Primer Pool	10 µL
Ion Ampliseq™ Sample ID Panel	1 µL
Total Per Reaction	**15 µL**

Table 2.2 *Target Amplification Master Mix.*

The DNA samples extracted in section 2.2.3, were diluted with nuclease free water to a 2 ng/µL concentration, and 15 µL 2X Single Primer Pool master mix was pipetted into a 0.2 mL PCR tube, followed by 5 µL DNA sample (10 ng total DNA). The sample was mixed thoroughly using 20 µL pipette a minimum of three times. The 20 µL sample in each PCR tube was centrifuged to spin down the

contents and eliminate any air bubbles. PCR was performed using the Veriti Dx 96 Well Thermal Cycler (Applied Biosystems™ by Thermo Fisher Scientific) to amplify the target region using the programme outlined in *Table 2.3*.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99 °C	2 min
Cycles: 22	Denature	60 °C	15 sec
	Anneal and Extend	99 °C	4 min
Hold	-	10 °C	**Hold**

Table 2.3 *Target Amplification Programme.*

Primer Sequence Partial Digestion

The PCR tubes were removed from the Veriti Dx 96 Well Thermal Cycler and centrifuged briefly. The samples were placed on ice. Two µL FuPa Reagent was added to each sample in the fume hood (total sample volume 22 µL). The sample was then vortexed and centrifuged briefly. The samples were placed in the Veriti Dx 96 Well Thermal Cycler using the programme outlined in *Table 2.4.*

Temperature	Time
50 °C	10 min
55 °C	10 sec
60 °C	20 min
10 °C	Hold

Table 2.4 *Primer Sequence Partial Digestion Programme.*

Ligation of Adaptors to the Amplicons

The Switch Solution and the DNA Ligase were thawed on ice. The Switch Solution was re-suspended at room temperature. The components outlined in *Table 2.5* were added in sequence to each sample, with each sample assigned a specific Ion Xpress™ Barcode Adapters (Ion Torrent™ by Thermo Fisher Scientific) as an identity marker.

Order of Addition	Component	Volume/Reaction
	DNA Sample	22 µL
1	Switch Solution	4 µL
2	IonCode™ Adapter	2 µL
3	DNA Ligase	2 µL
	Total Per Reaction	30 µL

Table 2.5 *Components for Ligation Reaction.*

Each sample was vortexed and centrifuged. The samples were placed in the Veriti Dx 96 Well Thermal Cycler using the programme outlined in *Table 2.5.*

Temperature	Time
22 °C	30 min
68 °C	5 min
72 °C	5 min
10 °C	Hold

Table 2.5 *Ligation Reaction Programme.*

Library Purification

Each sample was centrifuged briefly and pipetted into a 96 well PCR plate and placed on ice. 45 μL prewarmed and premixed Agencourt AMPure XP Reagent (Beckman Court ^{TM}) was added to each sample library and mixed by pipetting 5 times to ensure thorough mixing of the sample/beads. The samples were incubated for 5 min at room temperature. The 96 well plate was then placed on a Dynamag 96 slide magnetic rack (Invitrogen by Thermo Fisher Scientific) and incubated for 3 min or until the solution cleared. The supernatant was carefully removed and discarded ensuring the pellet was not disturbed.

One hundred and fifty μL 70% Ethanol was then added to each sample. The 96 well PCR plate was moved from side-to-side in the two positions on the magnetic plate to wash the beads. Once again, the supernatant was carefully removed and discarded, ensuring the pellet was not disturbed. This step was repeated once more. The beads were subsequently air dried for 3 min at room temperature.

Elution of Library

Upon removal of the 96 well PCR plate with the purified libraries from the magnet, 50 μL Low TE was added and mixed by pipetting half of the volume 5 times. The samples were then incubated at room temperature for 2 min. The 96 well PCR plate was placed on the magnetic plate for 2 min. All the supernatant from each sample on the 96 well PCR plate was removed to a LoBind® Eppendorf tube (Thermo Fisher Scientific).

2.3.3 Quantification of the Library by qPCR

The Ion Library TaqMan Quantitation Kit® (Ion Torrent™ by Thermo Fisher Scientific) was used to quantify sample libraries. The libraries were diluted 1:100 using nuclease free water. Three 10-fold serial dilutions of the E.coli DH10B Ion Control Library was made at 6.8 pM, 0.68 pM and 0.068 pM using nuclease free water. A qPCR negative was included (nuclease free water).

10 µL 2X Ion Library qPCR master mix was added to 1 µL Ion Library TaqMan® Quantitation Assay 20X per sample. A total 11 µL was pipetted into appropriate wells of a 96 well PCR plate. Technical duplicates were included for each sample, E-coli control, and negative control. 9 µL of nuclease free water served as the negative control. 9 µL of each standard (1, 2 and 3) was added to appropriate wells. Lastly 9 µL of each sample was pipetted into appropriate wells. In total, each well contained 20 µL. Each well was mixed by pipetting half the volume three times.

2.4 HPV Genotyping – Ion Torrent Next Generation Sequencing

2.4.1 Template Preparation

Templating of libraries was conducted using the Ion Chef ™ System (Ion Torrent™ by Thermo Fisher Scientific). The protocol, according to manufacturer's instructions (Ion 520™ & Ion 530™ Ext Kit – Chef), is briefly described below.

Preparation of the Ion Chef™ Instrument

The Ion Chef™ Instrument *(Figure 2.1)* (Ion Torrent™ by Thermo Fisher Scientific) was cleaned in conjunction with the Cancer Molecular Diagnostics (CMD), Centre for Laboratory Medicine and Molecular Pathology, SJH according to their validated protocols.

Ion 520™ Chef Reagents cartridges (Ion Torrent™ by Thermo Fisher Scientific) were removed from their respective packaging and allowed to remain at room temperature for 45 min to thaw and warm prior to usage.

Figure 2.1 *Ion Chef™ Instrument.*

Dilution of Library

The sample libraries were diluted to 100 pM with nuclease free water (n=51), with those of lower concentration remaining neat (n=16).

Library Pooling

Four pooled libraries were created (Pool 1 – n = 17, Pool 2 – n = 17, Pool 3 – n = 17; Pool 4 n = 16) using 5 µL from each sample library. Prior to loading on the machine, the library pools were further diluted to 33 pM (25 µL). Each pooled sample was pipetted to the bottom of the appropriate Ion Chef™ Library Sample Tube (barcoded tubes) cartridge (Ion Torrent™ by Thermo Fisher Scientific), on ice.

Loading the Ion Chef™ System (Ion Torrent™ by Thermo Fisher Scientific)
The protocol, according to manufacturer's instructions, is briefly described below.

Initially the tip rack was emptied. Correct positioning of the frame seal was conducted. New tip cartridges were placed. The PCR plate, Ion 520™ Chef Reagents cartridge inserted and Ion S5™ Chef Solutions cartridge were inserted. The Recovery Tubes and Recovery Station Disposable, Enrichment Cartridge and the Chip Adapter was inserted. The Ion Chef™ Library Sample Tube (barcoded tubes, each containing 25 µL of combined library) were inserted. Two Ion 520™ Chip (Ion Torrent™ by Thermo Fisher Scientific) was inserted in the Chip loading centrifuge. The deck scan was conducted and the Ion Chef™ run was initiated. In total, four Ion 520™ Chips were utilised in this study.

2.4.2 Sequencing

Sequencing was conducted using the Ion GeneStudio S5™ Semiconductor Sequencer (Ion Torrent™ by Thermo Fisher Scientific).

The protocol, according to manufacturer's instructions (Ion GenerStudio™ Instrument), is briefly described below.

Initialisation of the Sequencer was conducted in keeping with Manufacturer's guidelines. The Ion 520™ Chip Libraries were inserted into the Ion GeneStudio S5™ Semiconductor Sequencer (Ion Torrent™ by Thermo Fisher Scientific) for analysis (*Figure 2.2*).

Figure 2.2 *Ion GeneStudio S5™ Semiconductor Sequencer.*

2.4.3 Data Analysis

The sequencing data from each sample was aligned to a custom reference sequence containing 286 HPV genotypes in addition to the 4 human genes, *Appendix 2*. The sequencing data was analysed utilising the Burrows Wheeler Aligner (v.0.7.17) to conduct alignment analysis and extraction of alignment information. In addition, the normalised read counts were normalised to the mean read counts in the 4 human genes. PCR duplicate reads were not removed, in keeping with practices adopted in a similar study [234].

As a quality control (QC) standard, a cut off of an average 100 reads per samples from the human reference genes was instituted as the genotyping data was convincingly stable at this level, as illustrated in prior published studies [232, 234].

2.5 Targeted Somatic Mutation Analysis

Detection of targeted somatic mutations was conducted via single-molecule molecular inversion probe (smMIP) panel based next generation sequencing (NGS), with libraries sequenced using the Illumina NextSeq™ 550dx Instrument (Illumina Inc.) and analysed using bioinformatic software SEQNEXT (JSI Medical Systems).

SmMIPs are oligonucleotide probes containing an 'extension' and a 'ligation' arm bridged by a 'back bone'. The two arms of the probe are complimentary to a defined region of DNA that border the region of interest (ROI). The first step in the process is the capture phase which is composed of:

(i). Hybridisation of the probes to the genomic DNA

(ii). Extension of newly formed copied DNA to include the ROI by a polymerase

(iii). Ligation of the copied DNA with the ROI with the ligation probe

The next step involves treatment with exonuclease, resulting in removal of all linear DNA and unused/unligated smMIPs. The final stage is amplification of the ROI. The amplification step also amplifies the unique molecular identifier present in each circular molecule which allows for the identification of single molecule groups during post-sequencing [235-238]. The process is outlined in *Figure 2.3*.

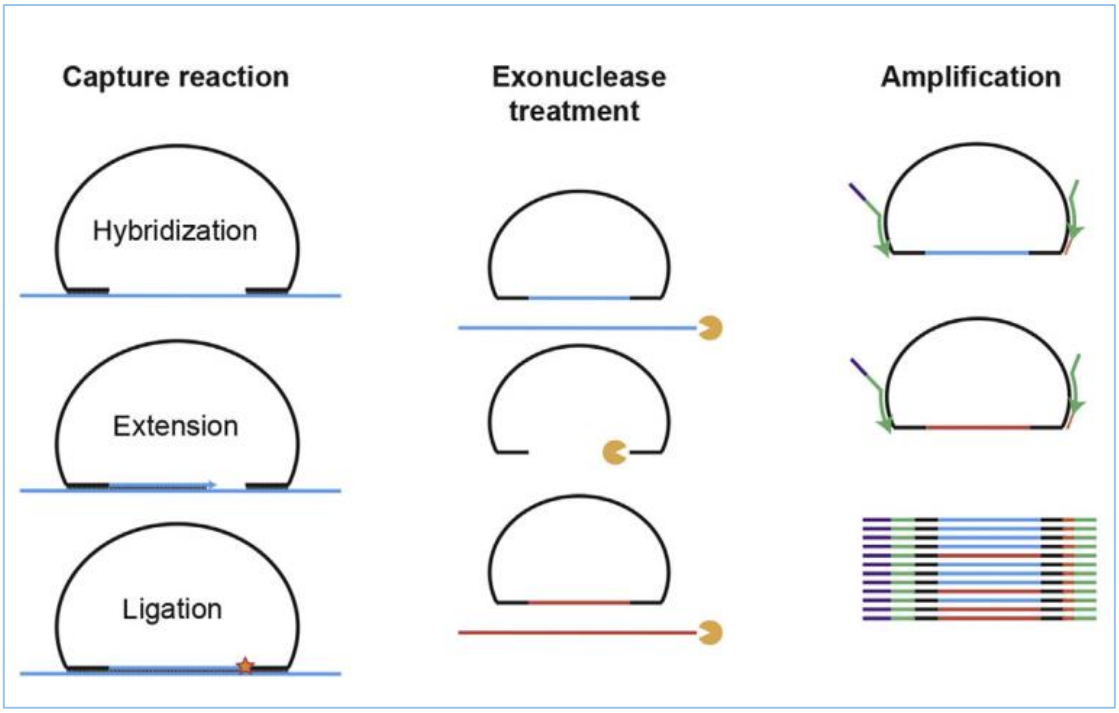

Figure 2.3 *Diagrammatic representation of smMIP library preparation.*
Source: Eijkelenboom et al. [236]

2.5.1 Somatic Mutation Detection via smMIP Panel

The CMD, Centre for Laboratory Medicine and Molecular Pathology, SJH *"Detection of pathogenic mutations using smMIP panels"* protocol was adhered to, and briefly described below. The somatic mutations analysed outlined in *Table 2.6.*

Gene	Minimum Coverage
BRAF	Exon 15/V600
EGFR	Exon 8, 19, 10, 21
ERBB2	Exon 20
KIT	Exon 8, 9, 11, 13, 14, 17
KRAS	Codons 12, 13, 61, 117, 146 (Exons 2, 3, 4)
MET	Exon 14
NRAS	Codons 12, 13, 61 (Exons 2, 3)
PDGFRA	Exons 12, 14, 18

Table 2.6 *Regions of Interest for the smMIP Panel.*

Abbreviations: EGFR, Epidermal Growth Factor Receptor; PDGFRA, Platelet Derived Growth Factor Receptor A

Library Preparation

Each library sample prepared was diluted to a concentration of 10 ng/uL and pipetted into a 96-well PCR plate. From the 67 samples, 59 had sufficient concentration levels suitable for further analysis.

1µL of the premade smMIP pool was added to each well followed by 7 µL of the premade hybridisation ligation mastermix. Each specimen was mixed by pipetting 5 times.

The 96-well PCR plate was sealed and placed in the centrifuge and spun at 1000 χg for 30 sec. The 96-well PCR plate was then loaded onto the C1000 Touch™ Thermal Cycler (Bio Rad Laboratories) using the pre-designed smMIP-hybridisation program.

Exonuclease treatment, barcoding, pooling and quantification

This step involved utilisation of the Hamilton® Microlab STARLet (Hamilton Company) *(Figure 2.4).*

Figure 2.4 *Hamilton® Microlab STARLet.*

The barcode plates and the iProof mastermix were removed from the freezer and thawed on the bench on ice. Once thawed, the barcode plates and the iProof mastermix were spun briefly in the centrifuge. The AMPure XP beads (Beckman Coulter™) were removed from the fridge and warmed on the bench for 20 min. The AMPure XP beads were subsequently vortexed until mixed thoroughly and the required volume was aliquoted into a 2 mL tube. The exonuclease mix was removed from the freezer and thawed on ice. Once all of the above steps were completed, the Hamilton® Microlab STARLet (Hamilton Company) was started utilising the "*smMIP lib prep"* predesigned protocol by CMD Laboratory. Once completed, the barcode plate was removed and tightly sealed. It was subsequently stored at -20 ℃. The tubes containing the library pool were quantified.

DNA Quantification Utilising the Invitrogen Qubit® Fluorometer

The extracted DNA was quantified utilising the Invitrogen Qubit® Flurometer and Qubit hsDNA and Quant-it Assay Kit (Qiagen) according to manufacturer's instructions, as described in *Section 2.2.4.*

2.5.2 Sequencing of Somatic Mutation Detected via smMIPs Panel

Sequencing of Somatic Mutation Detected via smMIPs Panel was conducted utilising the Illumina NextSeq^{TM} 550dx Instrument (Illumina Inc.).

The CMD *"Guidelines for the operation and maintenance of the Illumina NextSeq 550dx Platform"* protocol was adhered to, and briefly described below.
The Illumina NextSeq 500/550 Mid-Output v2.5 Kit (300 Cycles) (Illumina Inc.) was utilised.
The quantified libraries were diluted to 4 nM using the Elution Buffer (Illumina Inc.). The libraries were then combined to give a pooled library concentration of 4 nmol e.g. 10 µL of each 4 nmol library to be sequenced.

Library Preparation

The HT1 hybridisation buffer, Illumina Spike-in primers and PhiX (Illumina Inc.) were removed from storage in the -25 °C freezer to the -15 °C freezer and then thawed at room temperature. A Fresh Dilution of Sodium Hydroxide, NaOH was made by combining laboratory-grade water (800 µ L) and Stock 1.0 N NaOH (200 µL) in a microcentrifuge tube. The result is 1 mL 0.2 N NaOH. The microcentrifuge tube was then inverted several times. A fresh preparation of 200

µmol Tris-HCl pH 7 was made utilising the following: Stock 1 M – dilute 10 µL Tris-HCl + 40 µL H_2O.

Denaturing the Libraries

From a starting concentration of 4 nmol Library, 5 µL of the library was added to 5 µL 0.2 N NaOH in a microcentrifuge tube labelled 20 pmol library. The tubes were then vortexed briefly and centrifuged at 280 χg for 1 min and incubated at room temperature for 5 min. Subsequently, 5 µL 200 mmol Tris-HCl, pH 7 was added to the denatured libraries. The samples were then vortexed briefly and centrifuged at 280 χg for 1 min. The denatured libraries were then diluted to 20 pmol by adding 985 µL prechilled HT1. The samples were then vortexed briefly and centrifuged at 280 χg for 1 min and placed on ice. With the NextSeq 500/550 Mid-Output Kit v2.5 (Illumina Inc.), 97 µL of each denatured library solution was added to 1203 µL of the prechilled HT1 to enable a 1.5 pmol dilution. The resultant volume is 1.3 mL at 1.5 pmol. The solution was vortexed briefly and then pulse centrifuged.

Library and Primer Preparation

The illustrated volume of Phix Control v3 (Illumina Inc.) in *Table 2.7*, was added to the denatured libraries.

% Spike in	Volume 1.5 pmol library (µL)	Volume PhiX 1.5 pmol (µL)
10%	1170	**130**

Table 2.7 *Volume of Phix Control v3 added.*

Forward (R1), Reverse (R2) and Index primer were diluted in HT1 hybridisation buffer (Illumina Inc.) to a concentration of 10µmol, *Table 2.8*. The samples were vortexed and centrifuged briefly.

Tube Label	Volume 100µmol	Volume HT1 Hybridisation Buffer (µL)	Total Volume (µl)
20 F	5	**45**	50
20 R	5	**45**	50
22 Index	7	**63**	70

Table 2.8 *Forward (R1), Reverse (R2) and Index primer diluted in HT1 hybridisation buffer (Illumina Inc.) to a concentration of 10 µmol.*

Reagent Cartridge Loading

The v2 reagent cartridge was gently tapped to dislodge water from the base of the reagent cartridge. The cartridge was inverted 5 times to ensure adequate mixing of the reagents, followed by gentle tapping to reduce/minimise air bubbles. The foil

seal covering positions 20, 21, 22 and 10 was cleaned and pierced with a pipette tip. 1.3 mL 1.5 pmol library was pipetted into reservoir number 10 labelled Load Library. The appropriate volume of the custom primer was then added into the reagent cartridge. The v.25 flow cell (Illumina Inc.) as prepared in accordance with the stated guidelines.

2.5.3 Data analysis

Analysis was completed using JSI-SeqNext version 5.0.1 (JSI Medisystems, DE). Analysis was performed on fastq files generated. A minimum of 100 reads per target region was required to perform analysis and >300 reads with at least 50 reads on each strand to pass quality control. Mutations were confirmed if present in >2.5% of reads with a requirement that the ratio for detection be no greater than 4:1 in any direction. For single molecule groups, at least three reads with a minimum consensus coverage of 55% of reads was required and in cases in which a consensus of ≥30% could not be achieved, those cases were ignored.

2.5.4 HPV 16 Lineage

SAMtools (v1.16) was used to convert files from sam to bam format and generate consensus sequence files from for each sample. Multiple-sequence alignment of consensus sequences was carried out using MAFFT (v7.453). HPV16 sub-lineage references were retrieved from GenBank for each of the HPV16 sub-lineages.

Lineage	Taxon	Accession	Origin
A	A1	**K02718.1**	European Prototype
A	A2	**AF536179.1**	European German
A	A3	**HQ644236.1**	Europe
A	A4	**AF534061.1**	Europe/Asia
B	B1	**AF536180**	African 1a
B	B2	**HQ644298**	African 1b
C	C1	**AF472509**	African 2a
D	D1	**HQ644257**	Nort American 1
D	D2	**AY686579**	Asian American 1
D	D3	**AF402678**	Asian American 2

Table 2.9 *HPV lineages included in the study aligned against references sequences.*

Aligned consensus sequences and sub-lineage references were concatenated into a single fasta file to be used for phylogenetic analysis. RaxML (v2.0.8) was used to generate maximum likelihood trees using the GTRCAT model using the parameter below.

```
raxmlHPC-SSE3 -m GTRCAT -n HPV16_run4 -s HPV_consensus_genomes_sublineages.aln -p 12345 -N autoMRE \
 -f a -x 12345
```

The output of RaxML (Newick format tree) was visualised using Figtree (v1.4.4). The script nw2DistanceMatrix.py was used to generate a distance matrix and closestTaxon.py was used to determine the nearest sublineage to each sample.

```
python nw2DistanceMatrix.py --tree RAxML_bestTree.HPV16_run4 > distance_matrix.csv
python closestTaxon.py --dist_matrix distance_matrix.csv --taxon_list sublineage_names.txt
```

2.6 Statistical Analysis

The participant data collected was checked for consistency and completeness before being entered into the database software. STATA 16® was used for statistical analysis. Correlation values were determined via analysing continuous and categorical variables using logistic regression models and linear regression models. A probability of $p \leq 0.05$ was considered to represent a statistical correlation between the groups.

Chapter 3

Results

3.1 Study Population

Ethical approval was provided by the SJH/TUH joint research ethics committee (reference number 2020-09 (06)) and the RVEEH ethics committee meeting January 2020 (no reference number provided).

From the 01/01/2007 until the 31/12/2016, 86 patients were identified fitting the inclusion criteria. From patients identified, 4 were deemed lost to follow up and it was not possible to ascertain if they were alive or dead. Thirty five patients that met the criteria were deceased. Analysing the FFPE tumour blocks from this cohort, 1 FFPE tumour block had insufficient residual tumour within the specimen (> 100 tumour cells), with 1 FFPE tumour block not readily sourced.

At the time of the study on the 01/01/2020, 47 patients were alive based on assessment from medical charts and the Electronic Patient Record in SJH. A total of 35 patients provided a valid signed consent form by the deadline of the 30/06/2020. Twelve patients did not respond to the invitation. From the 35 patients who granted consent, 34 had sufficient tumour tissue within the FFPE tumour block. Diagrammatic representation of the breakdown of patients is illustrated in *Figure 3.1*.

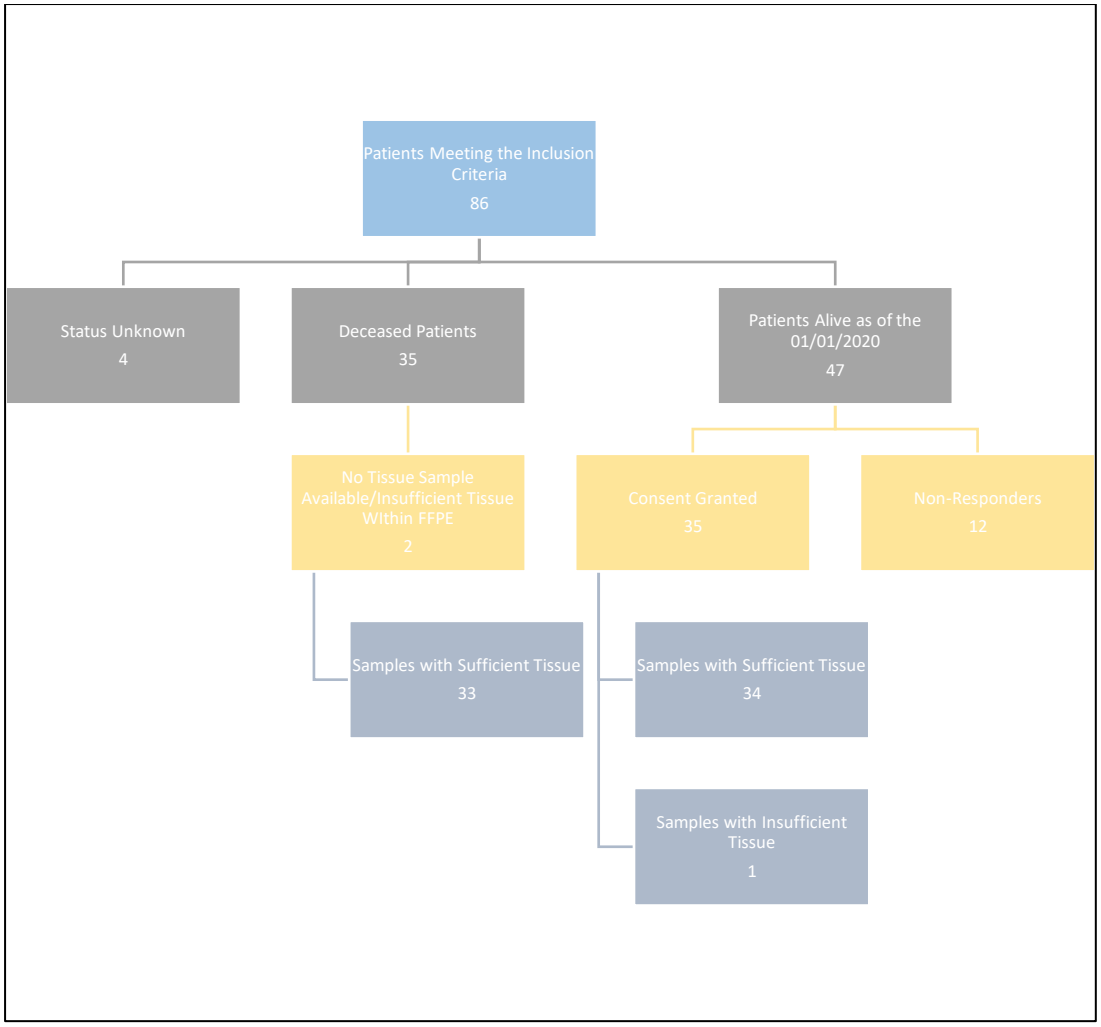

Figure 3.1 *Patients with HPV Positive OPSCC identified within the SJH Head and Neck Cancer database included in this study.*

3.2 Demographics of the Study Population

Patient characteristics at the time of diagnosis with OPSCC are outlined in *Table 3.1*. The most notable findings include a male predominance, 80.6 %, with an average age at diagnosis of 57.26 years.

Patient Characteristics	**n (%)**
Gender	
Male	54 (80.6)
Female	13 (19.4)
Age at diagnosis	
Range	41.49 – 83.12 years
Average age at diagnosis	57.26 years

Table 3.1 *Characteristics of Study Population: Age and Gender.*

Concerning the subsite within the oropharynx, *Table 3.2* illustrates the greatest proportion, 36 (53.73 %), were identified within the tonsil.

Tumour Site	n (%)
Base of Tongue	16 (23.88)
Tonsil	36 (53.73)
• **Right** • **Left**	18 (26.86) 18 (26.86)
Soft Palate	9 (13.43)
• **Right** • **Left** • **Not Stated**	2 (2.99) 5 (7.46) 2(2.99)
Vallecula	1 (1.5)
Vallecula, Base of Tongue, Epiglottis	1 (1.5)
Oropharynx, Not Otherwise Specified	4 (6)
• **Right** • **Left** • **Not Stated**	1 (1.5) 1 (1.5) 2 (3)

Table 3.2 *Subsite of the Primary Tumour within the Oropharynx.*

Within the study cohort, patients were diagnosed with OPSCC from 01/01/2007 to 31/12/2016. Cancer cases diagnosed in 2007 and 2008 were staged using the American Joint Committee on Cancer/ Union for International Cancer Control (AJCC/UICC) 6th Edition as per staging criterion at that time period. Subsequent cases were staged using the AJCC/UICC 7th Edition. In total 5 of the 67 cases were staged using the AJCC/UICC 6th Edition criteria. Comparing the 6th and 7th Editions, there was no discernible difference introduced pertaining to the staging criteria for tumours arising within the oropharynx. Thus, the 5 cases staged with the AJCC/UICC staging manual 6th Edition were restaged using the AJCC/UICC TNM 7th Edition. The TNM and AJCC/UICC Staging criteria are outlined in *Appendix 3*.

The results from this study illustrate that T2 N2 M0 was the prevailing clinical stage at diagnosis, with a corresponding pathological TNM stage of pTX pN2 pMX, *Table 3.3*. This corresponds to a Clinical Stage of IVA, *Table 3.4*. In context, the majority of participants had an oropharyngeal tumour of between 2 cm and 4 cm, with nodal disease confined to the head and neck region with no metastasis. Of note, only 3 patients (4.48 %) had clinically diagnosed metastatic disease at time of primary diagnosis on radiological imaging, *Table 3.4*.

TNM 7th Edition Staging Classification of Oropharyngeal Cancers			
Clinical		**Pathological**	
T		**T**	
Not Stated	3	**Not Stated**	2
X	-	**X**	45
1	9	**1**	8
2	31	**2**	9
3	14	**3**	0
4	10	**4**	1
Not Subcategorised	5		
4a	3		
4b	2		
N		**N**	
Not Stated	1	**Not Stated**	2
0	13	**0**	3
1	11	**1**	7
2	37	**2**	13
2a	-	**2a**	5
2b	-	**2b**	8
2c	-	**2c**	0
3	5	**3**	0
X	-	**X**	40
M		**M**	
Not Stated	1	**Not Stated**	2
0	63	**0**	0
1	3	**1**	0
X	-	**X**	63

Table 3.3 Continued			
		Staging post-Neoadjuvant Therapy	
		yTX	1
		yT1	1
		yN0	1
		yN1	1
		yMx	2

Table 3.3 *TNM Staging Classification of the Primary Oropharyngeal Cancers.*

AJCC/UICC 7th Edition Clinical Staging of Tumours	
Not Stated	3
I	2
II	10
III	14
IVA	34
IVB	6
IVC	3

Table 3.4 *AJCC/UICC Staging of Tumours for the Primary Oropharyngeal Cancers in this study.*

The treatment regimen received by each patient based at the time of primary diagnosis is displayed in *Table 3.5*. Sixty-two patients (92.54 %) were treated with the primary aim of curative intent. Five patients (7.46 %) were treated with a palliative regimen composed of chemotherapy or combined CRT. Twenty three of the 62 patients treated along the curative pathways (37.1 %) had a surgical intervention, with a total of 58 (93.55 %) receiving chemotherapy and/or immunotherapy.

Treatment Regimens Received	
Palliative Care Treatment	5
Chemotherapy	1
Chemotherapy/Radiotherapy	4
Chemotherapy Regimens For Those Receiving Palliative Care Treatment	
Platinum Based Therapy (Carboplatin/Cisplatin)	4
Induction Docetaxel, Cisplatin, Fluorouracil followed by Carboplatin	1
Curative Intent Treatment	62
Surgical Resection of Primary Tumour	7
Chemotherapy/Radiotherapy	38
Surgical Resection of Primary Tumour + Chemotherapy/Radiotherapy	16
Unknown	1
Chemotherapy	
Chemotherapy Regimens	54
Platinum Based Therapy (Carboplatin/Cisplatin)	42
Cetuximab	3
Docetaxel, Cisplatin, Fluorouracil followed by Pegfilgrastim	1
Docetaxel, Cisplatin, Fluorouracil followed by Carboplatin	3
Cetuximab followed by Carboplatin and Fluorouracil	1
Cisplatin followed by followed by Cetuximab and Fluorouracil	1
Unknown	3

Table 3.5 *Treatment Regimens Received Post Original Primary Oropharyngeal Cancer Diagnosis.*

Regarding the lifestyle factors of smoking and alcohol, 25 (37.31 %) of patients were active smokers at time of primary diagnosis, with 24 (35.82 %) classified as former smokers. The time period from cessation of smoking to the time of diagnosis ranged from 1 to 40 years, with a mean period of 14 years. Unfortunately, there was limited documentation available pertaining to pack year history, and thus such data could not be assessed in this study.

Concerning alcohol consumption, 50 (74.63 %) of patient consumed alcohol at the time of primary diagnosis, with 20 (29.85 %) classified as consuming alcohol in excess (defined as > 14 units/week for a male; > 11 units/week for a female). Four patients were defined as currently not consuming alcohol, but who previously consumed alcohol in excess. *Table 3.6* displays the lifestyle factors identified in the study population.

Patient Characteristics	n (%)
Smoking Status	
Unknown	3 (4.48)
Non-Smoker	15 (22.39)
Former-Smoker	24 (35.82)
Smoker	25 (37.31)
Alcohol Consumption	
Current consumer of alcohol	50 (74.63)
Never consumed alcohol	6 (8.96)
Prior consumer of alcohol	5 (7.46)
Unknown	6 (8.96)
Prior Alcohol Consumption in Excess	4 (6.56)
Alcohol Consumption in Excess	20 (32.79)

Table 3.6 *Characteristics of Study Population: Smoking and Alcohol Consumption*[1].

[1] Alcohol consumption in excess was defined as consuming > 14 units of alcohol/week for a male; > 11 units/week for a female.

3.3 HPV Positive OPSCC with Locoregional Recurrence and/or Distant Metastasis

Eleven patients were deceased at the time of the study, the date at which death occurred unknown. The remaining 56 patients were deceased, with a known date of death, or alive at the time of this study and had consented to participate. The mean and median follow-up period for the 56 patients was 8.67 years and 6.92 years respectively.

Illustrated in *Table 3.7* is the number of patients, 19 (28.36 %), with known LR and/or DM of their primary HPV OPSCC. Excluding those for whom adequate follow-up had not been sourced (n = 63), the figure rises to 30.16%. From the 19 cases of LR and/or DM, 12 (63.16 %) were defined as LR, with 7 cases (36.84%) arising in regions outside the head and neck (DM). The overall rate of LR was 19.05 %, and of DM, 11.11 %. Displayed in *Table 3.8 i*s the site of LR and/or DM in cases of HPV positive OPSCC. Identified 20 sites of LR and/or DM in 19 patients with one patient having more than one site of LR and/or DM.

HPV Positive OPSCC with Locoregional Recurrence and/or Distant Metastasis	(%)
Patient Follow Up Data Sourced	
No	4
Yes	63
Locoregional Recurrence and/or Distant Metastasis (n = 63)	
No	44 (69.84)
Yes	19 (29.69)

Table 3.7 *HPV Positive OPSCC with LR and/or DM.*

Sites of Locoregional Recurrence and/or Distant Metastasis	(%)
Oral Cavity – Local	5 (26.32)
Posterior Nasal Space	1 (5.26)
Lung – Bronchus/Parenchyma	6 (31.58)
Vertebral	2 (10.53)
Neck	6 (31.58)

Table 3.8 Sites of local cancer LR and/or DM post original treatment of primary HPV positive OPSCC.

Nineteen (100 %) of cases showing LR and/or DM post original treatment were diagnosed via radiological investigations, with 16 (84.21 %) having confirmatory histology from biopsy or surgical resection of the site of recurrence. This signifies that not all cases had confirmatory histopathological diagnosis of metastasis.

From those cases with LR and/or DM, 18 (94.74 %) had died from their disease, with one (5.26 %) alive at the time of completion of analysis. The cut-off date for data analysed relating to patient follow up was the 30/06/2022. From the entire study cohort 7 (11.29 %) patients had a new incidental primary cancer deriving from outside the head and neck. This was not identified in any of the cases with LR and/or DM. Incidental primaries identified included 3 lung adenocarcinomas, 2 prostate adenocarcinomas, 1 lung squamous cell carcinoma (p16 negative), and a single case of primary p16 negative HNSCC.

3.4 Correlation of Patient Demographics with HPV Positive OPSCC with Locoregional Recurrence and/or Distant Metastasis

Comparing the gender of the patients with LR and/or DM, 16 (84.21%) were male, with the remaining 3 cases female (15.79 %). Comparing these results to the gender difference in the non-recurrence cohort: Male, 35 (79.55 %); Female, 9 (20.45 %). The average age at primary diagnosis for those who subsequently went on to develop LR and/or DM was 54.16 years, in contrast to 58.16 years in the cohort who did not develop LR and/or DM.

As stated in *Section 3.3*, regarding the site of the recurrence/metastasis, 63.16 % were LR, and 36.84 % were DM. Logistic regression model illustrated in *Table 3.9* shows that no statistically significant correlation was found between the site of LR and/or DM and baseline patient demographics including age and gender.

Baseline Patient Demographics with the Site of the Locoregional Recurrence and/or Distant Metastasis				
n = 16	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
Age of Diagnosis (Years)	0.9627694	0.634059	**0.565**	0.8461824 to 1.09542
Sex	4.706127	7.067006	**0.302**	0.2479978 to 89.30577
Current & Former Smoker	1	-	-	-
Current/Previous Excessive Alcohol Consumption	3.598194	4.582427	**0.315**	0.2965168 to 43.66363

Table 3.9 *Logistic Regression Model Correlating Baseline Patient Demographics with the Site of LR and/or DM.*

From the 19 cases of LR and/or DM, 11 (57.89 %) of those patients had a primary tonsillar SCC as displayed in *Figure 3.2*. Of note, 11 of the 36 (30.55 %) patients with primary tonsillar HPV positive SCC developed LR and/or DM, in contrast to 4 (25 %) with base of tongue, 3 (33 %) with a soft palate and 1 (25 %) with an oropharynx, NOS primary tumour.

From the 44 cases without LR and/or DM, 22 (50 %) of cases originated within the tonsil, 12 (27.27 %) the base of tongue, 5 (11.36 %) the soft palate, and 3 (6.82 %) the oropharynx, NOS and 2 (4.45 %) the vallecula.

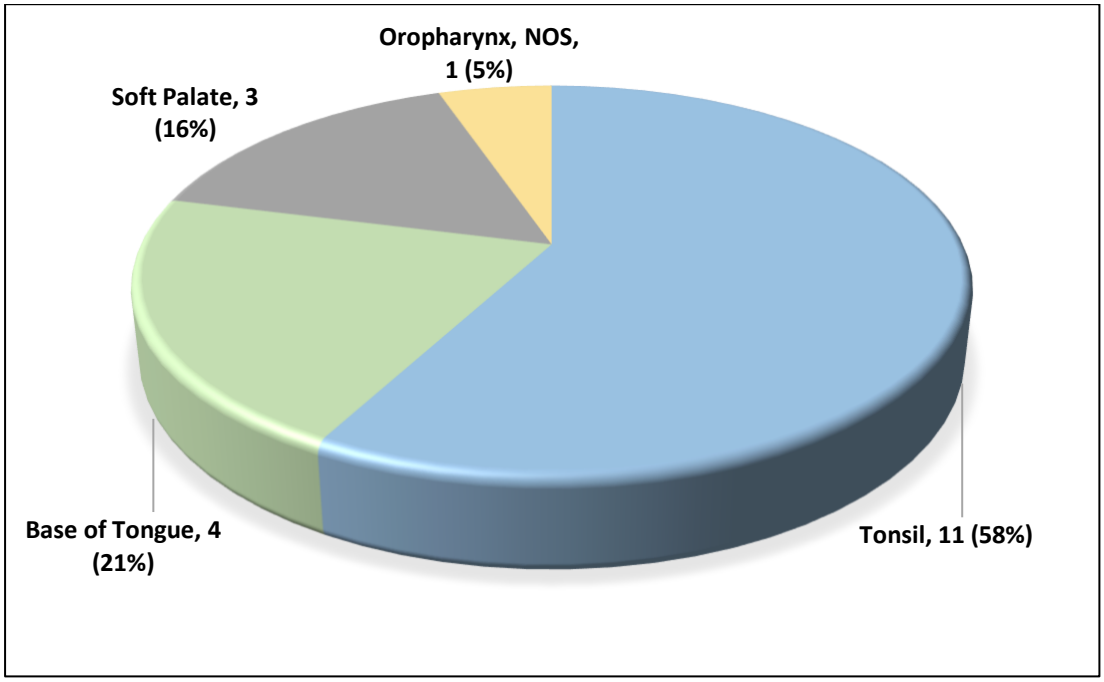

Figure 3.2 *Site of Primary SCC within the Oropharynx in Cases with LR and/or DM.*

A logistic regression model illustrated in *Table 3.10* illustrates no statistically significant correlation was identified between primary tumour site and the development LR and/or DM when age, gender, and smoking status were accounted for.

Primary Tumour Site with Locoregional Recurrence and/or Distant Metastasis				
n = 63	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
Base of Tongue	0.6570217	0.4711535	**0.558**	0.161131 to 2.679048
Oropharynx	0.6792755	0.8492677	**0.757**	0.0585905 to 7.872563
Soft Palate	1.124803	0.9505793	**0.889**	0.2146444 to 5.894314
Tonsil	1	-	**-**	-
Age at Diagnosis (Years)	0.9463228	0.0343165	**0.128**	0.8813983 to 7.640277
Gender	1.655358	1.291724	**0.518**	0.1282127 to 7.640277

Table 3.10 *Logistic Regression Model Correlating Site of Primary Tumour with Development of LR and/or DM When Age and Gender Were Accounted For.*

Reviewing the AJCC/UICC clinical stage at primary diagnosis for the cases with identified LR and/or DM, the staging was as follows: Stage I: 0; Stage II: 1 (5.26 %); Stage III: 3 (15.79 %); Stage IVA: 12 (63.16 %); Stage IVB: 3 (15.79 %). Findings are illustrated in *Figure 3.3.* A statistically significant correlation was identified between the AJCC/UICC Stage of Primary OPSCC in cases with LR and/or DM (p = 0.01), outlined in *Table 3.11*. This illustrates that a higher stage at diagnosis is correlated with LR and/or DM.

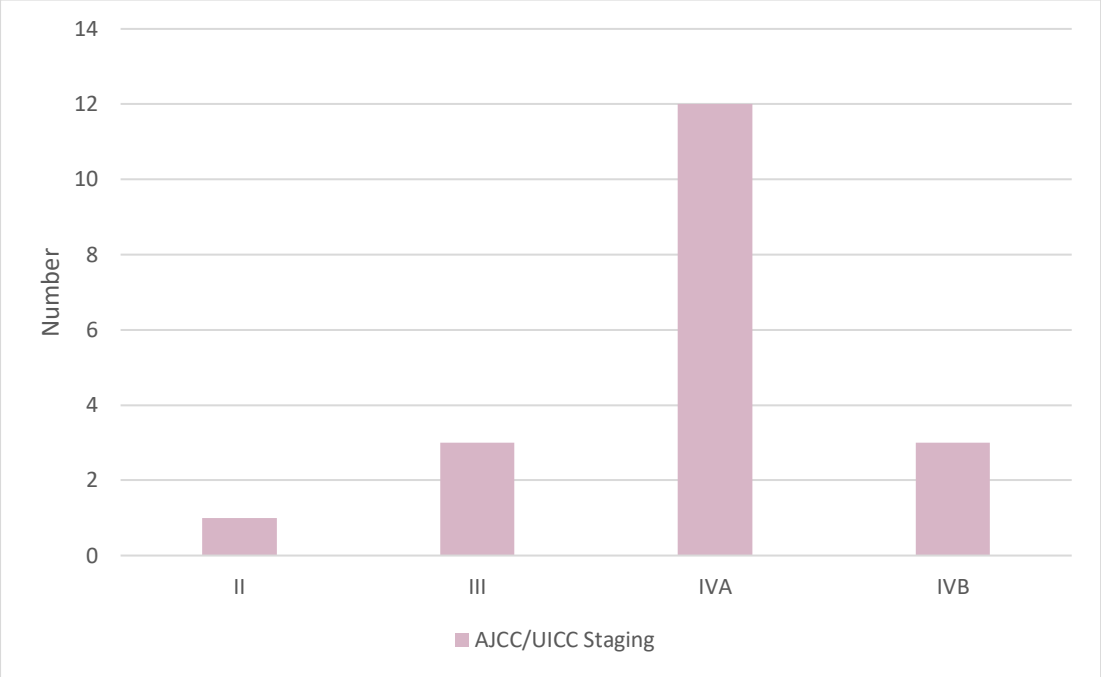

Figure 3.3 *AJCC/UICC Stage of Primary OPSCC in Cases with LR and/or DM.*

AJCC/UICC Stage with Locoregional Recurrence and/or Distant Metastasis				
n = 50	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
LR and/or DM	6.026082	4.224536	**0.01**	1.52513 to 23.8102
Age of Diagnosis (Years)	0.9765591	0.0310186	**0.455**	0.9176176 to 1.039287
Gender	0.9705978	0.742983	**0.969**	0.2164975 to 4.351366
Current & Former Smoker	0.4851874	0.3309754	**0.289**	0.1274248 to 1.847417
Current/Previous Excessive Alcohol Consumption	0.3031531	0.2013563	**0.072**	0.0824686 to 1.114386

Table 3.11 *Logistic Regression Model Correlating AJCC/UICC Stage with LR and/or DM When Age, Gender, Smoking Status and Alcohol Were Accounted For.*

Examining the original treatment regimen received for the cases which subsequently developed LR and/or DM, 6 (31.58 %) had underwent surgical intervention, with 13 (68.42 %) receiving CRT. Note however, that all patients who had surgical resection of their disease recurrence had follow-up combined CRT.

Analysing the 6 patients who had a surgical resection of the primary tumour, 2 (33 %) had recurrence at that localised site. Reviewing the 6 patients who underwent a neck dissection at time of primary diagnosis, 3 (50 %) patients had subsequent neck nodal disease at time of recurrence. Two (11.76 %) patients who had nodal involvement post treatment for primary tumour did not have a surgical neck dissection at time of primary diagnosis.

Table 3.12 shows that no statistically significant correlation was found between LR and/or DM and treatment type received for primary tumour when age and gender were accounted for. It is noteworthy that within this model of analysis, whilst the correlation between age at diagnosis and the development of LR and/or DM does not reach a statistical significance, there is a strong correlation signifying the younger the age at diagnosis, the increased likelihood of LR and/or DM ($p = 0.057$). A logistic regression model illustrated in *Table 3.13* shows that no statistically significant correlation was found between treatment type received for primary tumour and the site of LR and/or DM received when age and gender were accounted for.

Treatment Regimen Received for Primary Tumour with Locoregional Recurrence and/or Distant Metastasis				
n = 59	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
Chemotherapy	0.6694005	0.75616	0.722	0.0731412 to 6.126463
Radiotherapy	1	-	-	-
Surgery	0.5367695	0.4726132	0.480	0.0955706 to 3.014749
Neck Dissection	2.253636	1.75828	0.298	0.4884037 to 10.39893
Age of Diagnosis (Years)	0.9314697	0.347871	0.057	0.8657238 to 1.002209
Gender	1.487224	1.301689	0.650	0.2622606 to 8.267767

Table 3.12 *Logistic Regression Model Correlating LR and/or DM with Treatment Type Received for Original Tumour When Age and Gender Were Accounted For.*

Treatment Type Received for Primary Tumour with the Site of Locoregional Recurrence and/or Distant Metastasis				
n = 17	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
Chemotherapy	1	-	-	-
Radiotherapy	1	-	-	-
Surgery	8391424	2.1×10^{10}	0.995	0 to -
Neck Dissection	2.191681	3.323684	0.605	0.1121838 to 42.81781
Age of Diagnosis (Years)	0.9811394	0.0515269	0.717	0.8851723 to 1.087511
Gender	1.5×10^{7}	3.75×10^{10}	0.995	0 to -

Table 3.13 *Logistic Regression Model Correlating Treatment Type Received for Original Tumour with the Site of LR and/or DM with When Age and Gender Were Accounted For.*

Examining the correlation lifestyle factors of smoking and alcohol consumption with LR and/or DM yielded very interesting results. *Figure 3.4* displays the smoking status of each patient categorised based on their recurrence status. Eighteen of the 19 (94.74 %) cases who developed LR and/or DM were former or were currently smoking at the time of diagnosis, in contrast to 30 (68.18 %) of those who did not develop disease recurrence.

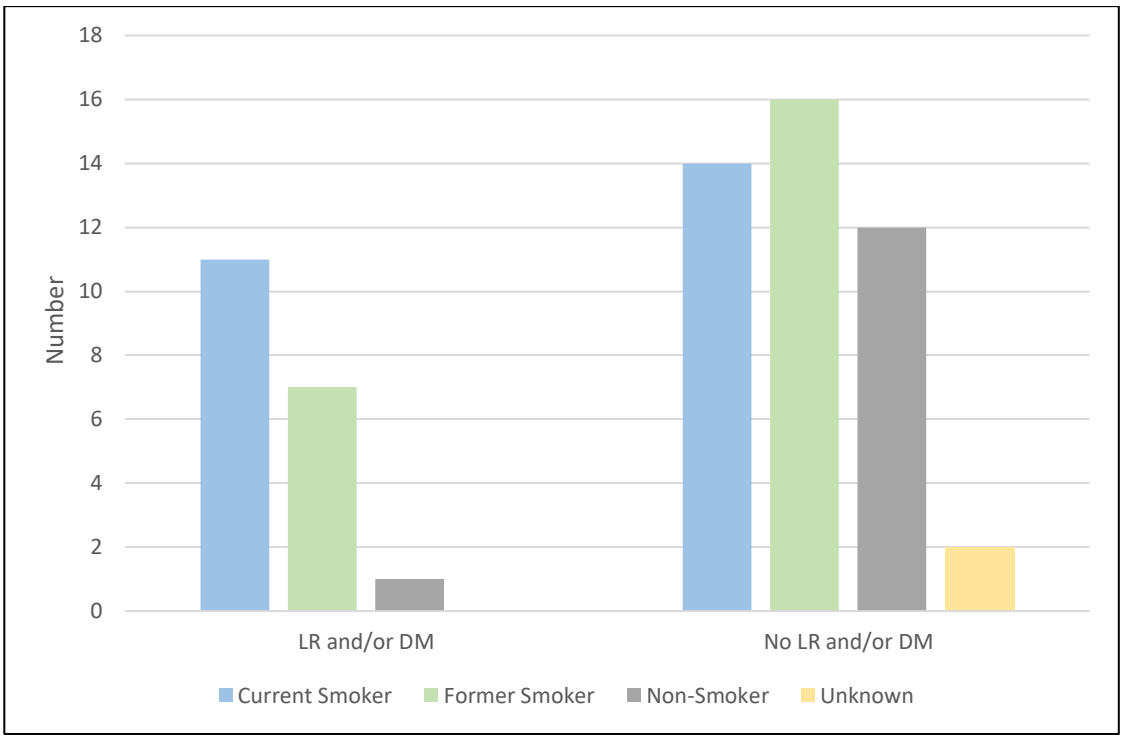

Figure 3.4 *Smoking Status of Patients and LR and/or DM of primary OPSCC.*

Figure 3.5 displays the alcohol consumption of each patient categorised based on their recurrence status. Seventeen of the 19 (89.47 %) cases who developed LR and/or DM were consuming alcohol at the time of diagnosis, with the remaining 2 patients (10.53 %) both defined as previously consuming alcohol in excess but abstinent at time of diagnosis. In total 10/19 (52.62 %) and 12/19 (63.16 %) of those with LR and/or DM consume or were consuming alcohol in excess at the time

of primary diagnosis. Drawing comparisons to those who did not develop further disease progression post initial treatment, 33 (75 %) were consumers of alcohol at time of diagnosis, with 2 (4.54 %) classified as prior consumers of alcohol. In total 10/44 (22.72 %) and 12/44 (27.27 %) of those with no disease progression consume or were consuming alcohol in excess at the time of primary diagnosis.

As illustrated in a logistic regression model in *Table 3.14*, there was no statistically significant correlation identified between age, gender, or current/former smoking status with LR and/or DM. However, a statistically significant correlation is evident between prior or current excessive alcohol consumption and LR and/or MD when age, gender and smoking status are accounted for (n = 51, Odds Ratio 4.563, p = 0.038, 95% C.I. 1.092 – 19.079).

Current/Previous Excessive Alcohol Consumption with Locoregional Recurrence and/or Distant Metastasis				
n = 51	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
Current/Previous Excessive Alcohol Consumption	4.563368	3.330626	**0.038**	1.0091506 to 19.07852
Age of Diagnosis (Years)	0.9244787	0.431835	**0.093**	0.8435994 to 1.013112
Gender	0.9670346	0.8922943	**0.971**	0.1584996 to 5.900051
Current & Former Smoker	4.527938	5.210541	**0.189**	0.4746538 to 43.19404

Table 3.14 *Logistic Regression Model Correlating Prior or Current Excessive Alcohol Consumption with LR and/or DM with Age, Sex and Smoking Status Accounted For.*

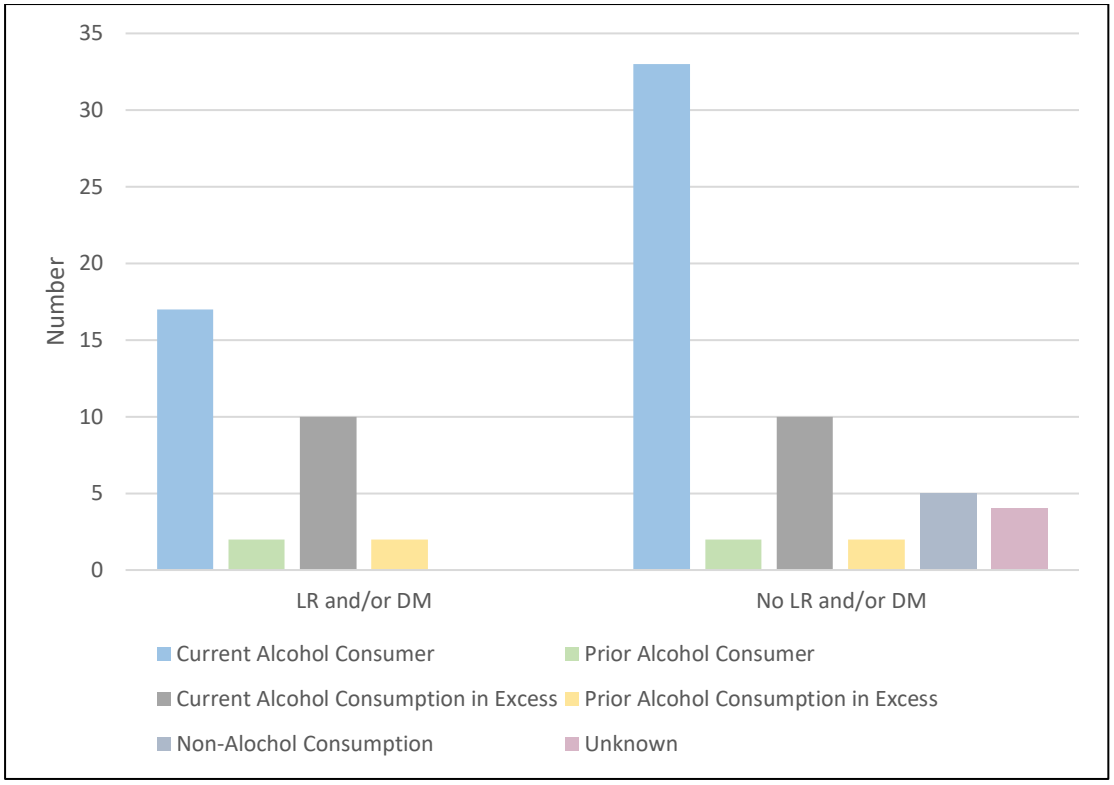

Figure 3.5 *Alcohol Consumption Status of Patients and LR and/or DM of primary OPSCC.*

The final aspect to examine is in relation to the impact on patient demographics and the DFI. One patient developed a LR while receiving treatment, and thus was not included in analysis for DFI as primary treatment was not completed. The average time period from completion of primary treatment regimen to diagnosis of LR and/or DM was 1.5 years, with a median of 0.74 years and a range from 0.3 to 4.35 years.

Table 3.15 shows a linear regression model, which illustrates no correlation was identified between baseline patient demographics including age, gender, current/prior excessive smoker, and current/prior excessive alcohol consumption with the DFI. *Table 3.16, 3.17 and 3.18* illustrates no statistically significant

correlation was identified between treatment received for primary tumour, the site of the primary tumour and the development of LR and/or DM with the DFI.

Baseline Patient Demographics with Disease Free Interval				
n = 16	**Coefficient**	**Standard Error**	**p Value**	**95% Confidence Interval**
Current/Previous Excessive Alcohol Consumption	-11.56273	7.097992	**0.129**	-27.02793 to 3.902462
Age of Diagnosis (Years)	-0.3408742	0.3616355	**0.364**	01.12881 to 0.4470618
Gender	2..20163	8.044592	**0.789**	-15.32603 to 19.72929
Current & Former Smoker	0	-	**-**	-

Table 3.15 *Linear Regression Model Correlating Basic Patient Demographics with DFI.*

Primary Treatment Received with Disease Free Interval				
n = 18	**Coefficient**	**Standard Error**	**p Value**	**95% Confidence Interval**
Radiotherapy	0	-	-	-
Chemotherapy	18.62396	10.88779	**0.113**	-5.098492 to 43.34642
Surgery	17.13117	8,919029	**0.079**	-2.301729 to 36.56406
Neck Dissection	0.6267701	6.918986	**0.929**	-14.4484 to 15.70195
Age of Diagnosis (Years)	-0.105608	0.285582	**0.718**	-0.7278377 to 0.5166216
Gender	11.86201	8.549457	**0.181**	-6.765657 to 30.48967

Table 3.16 *Linear Regression Model Correlating Primary Treatment Received with DFI When Age and Gender Were Accounted For.*

Locoregional Recurrence and/or Distant Metastasis with Disease Free Interval				
n = 16	**Coefficient**	**Standard Error**	**p Value**	**95% Confidence Interval**
Local and/or Distant Recurrence	0/069366	6.984525	**0.262**	-7.303469 to 23.4422
Age of Diagnosis (Years)	-0.2798467	0.3605844	**0.454**	-1.073488 to 0.5137942
Sex	-0.354367	8.237333	**0.966**	-18.48461 to 17.77588
Current & Former Smoker	0	-	**-**	-
Current/Previous Excessive Alcohol Consumption	-13.72638	7.247165	**0.085**	-29.67728 to 2.224521

Table 3.17 *Logistic Regression Model Correlating LR and/or DM with DFI When Baseline Patient Demographics Were Accounted For.*

Primary Tumour Site with Disease Free Interval				
n = 18	**Coefficient**	**Standard Error**	**p Value**	**95% Confidence Interval**
Base of Tongue	13.77428	13.18438	**0.317**	-14.95201 to 42.50058
Oropharynx	0	-	**-**	-
Soft Palate	1.786455	15.00904	**0.907**	-30.91544 to 34.48835
Tonsil	-0.9000735	12.48359	**0.944**	-28.09949 to 26.29934
Age at Diagnosis (Years)	-0.3333296	0.3160663	**0.312**	-1.021979 to 0.3553197
Gender	-1.467725	23.58478	**0.396**	-30.61055 to 72.1631

Table 3.18 *Linear Regression Model Correlating Site of the Primary Tumour with DFI.*

3.5 Human Papillomavirus Genotype

3.5.1 HPV Genotype

The Ion GeneStudio S5 System is a semiconductor-based NGS utilised in this study to identify HPV genotypes in p16 positive HNSCC. The sequencing data from each sample was aligned to a custom reference sequence containing 286 HPV genotypes, in addition to the 4 human genes, *Appendix 2*. As discussed in *Chapter 2*, a QC standard of an average of 100 reads from the human reference genes per samples was instituted. One sampled failed to meet QC standards, this case was excluded from the study. The full read count is outlined in *Appendix 4.*

From the 67 samples in which HPV genotyping was conducted, 1 did not meet the QC standard in terms of absent/low reads detected from the 4 human reference genes. Three samples did not have >100 reads in one or more HPV genotypes and were thus excluded from further analysis. Thus, data extracted from this study is from 63 samples (n = 63). From the 63 samples, 59 detected one HPV genotype. From the 63 samples, 4 samples had co-infection with two HPV genotypes. One case had co-infection of HPV 16 and 39 with almost identical reads, indicating an almost parallel infection. The remaining three cases had a predominant genotype with a definitive secondary less prevalent genotype.

The genotype with the highest read count was HPV 16, 59/63 (93.65%). The remaining three cases, 3/63 (4.48%), aligned to HPV 33 (4.76%) and one case aligned to HPV 35 (1.59%). Removing the 4 cases with dual infection: HPV 16 was detected in 57/59 (96.61 %) and HPV 33 in 2/59 (3.39 %). The HPV genotypes identified from this study are illustrated in *Table 3.19.*

HPV Genotypes Identified from the Ion Torrent Next Generation Sequencing (%)	
n = 63	
Samples with One HPV Genotype Detected	
n = 59	
HPV 16	**57 (96.61)**
HPV 33	**2 (3.39)**
Samples with Two HPV Genotype Detected	
n = 4	
Primary Subtype	**Secondary Subtype**
1. HPV 16	**HPV 18**
2. HPV 16	**HPV 33**
3. HPV 33	**HPV 16**
4. HPV 35	**HPV 16**

Table 3.19 *HPV Genotypes Identified From the Ion Torrent Next Generation Sequencing.*

As displayed, HPV 16 was the predominant genotype identified and thus statistical analysis was conducted pertaining to this genotype. Logistic and linear regression models illustrated in *Tables 3.20, 3.21, 3.22 and 3.23* show no statistically significant correlation was identified between the HPV 16 Genotype and LR and/or DM (*Table 3.20)*, the site of LR and/or DM (*Table 3.21)*, DFI (*Table 3.22)* and the subsite of primary tumour (*Table 3.23)*, when factors including age, gender, smoking and alcohol consumption in excess were accounted for.

An interesting observation was identified when utilising linear regression model to asses if a statistically significant correlation could be identified between the HPV 16 genotype and DFI, accounting for factors including age, gender, smoking and alcohol consumption in excess. Outlined in *Table 3.22*, a statistically significant correlation was identified between the DFI and a prior or current excessive alcohol consumption when the exact HPV genotype is known (n=15, coef. -20.323, p = 0.033, 95% C.I. -36648 – -1.998).

HPV 16 Genotype with Locoregional Recurrence and/or Distant Metastasis				
n = 48	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
HPV 16	0.96065	1.322659	**0.976**	0.064509 to 14.28832
Age of Diagnosis (Years)	0.9298191	0.0428592	**0.123**	0.8477105 to 1.019881
Gender	0.8780069	0.8293834	**0.890**	0.1378591 to 5.591914
Current & Former Smoker	4.0034277	4.733437	**0.235**	0.4046194 to 40.22394
Current/Previous Excessive Alcohol Consumption	4.009026	2.968393	**0.061**	0.93926 to 17.111165

Table 3.20 *Logistic Regression Model Correlating HPV 16 Genotype with LR and/or DM, When Age, Gender, Smoking and Alcohol Consumption in Excess Were Accounted For.*

HPV 16 Genotype with The Site of Locoregional Recurrence and/or Distant Metastasis				
n = 14	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
HPV 16	1	-	-	-
Age of Diagnosis (Years)	0.8819461	0.0819003	**0.176**	0.7351852 to 1.058004
Gender	5.160253	8.646457	**0.327**	0.1933785 to 137.7
Current & Former Smoker	1	-	-	-
Current/Previous Excessive Alcohol Consumption	0.6224174	1.08867	**0.786**	0.201951 to 19.18305

Table 3.21 *Logistic Regression Model Correlating HPV 16 Genotype with LR and/or DM Site When Age, Gender, Smoking and Alcohol Consumption in Excess Were Accounted For.*

HPV 16 Genotype with Disease Free Interval				
n = 15	**Coefficient**	**Standard Error**	**p Value**	**95% Confidence Interval**
HPV 16	29.02135	15.36379	**0.088**	-5.211312 to 63.24501
Age of Diagnosis (Years)	-0.7412432	0.4018491	**0.095**	-1.636619 to 0.1541325
Gender	2.684716	7.645197	**0.733**	-14.34985 to -19.71928
Current & Former Smoker	1	-	**-**	-
Current/Previous Excessive Alcohol Consumption	0.6224174	1.08867	**0.033**	-38.64798 to -1.99856

Table 3.22 *Linear Regression Model Correlating Disease Free Interval with HPV 16 Genotype When Age, Gender, Smoking and Alcohol Consumption in Excess Were Accounted For.*

Primary Tumour Subsite with HPV 16 Genotype				
n = 41	**Odds Ratio**	**Standard Error**	**p Value**	**95% Confidence Interval**
Base of Tongue	1	-	**-**	-
Oropharynx	1	-	**-**	-
Soft Palate	0.6897038	0.8573596	**0.765**	0.060332 to 7.884559
Tonsil	1	-	**-**	-
Age at Diagnosis (Years)	0.9949786	0.0670788	**0.940**	0.8718223 to 1.135532
Gender	1.090402	1.343275	**0.944**	0.0974956 to 12.19519

Table 3.23 *Logistic Regression Model Correlating Subsite of The Primary Tumour with HPV 16 Genotype When Age and Gender Were Accounted For.*

Concerning the 4 samples with dual HPV genotypes detected, all patients were male with age range from 40 to 60 years. The average age at diagnosis was 49.5 years.

The subsite within the oropharynx included: tonsil (2), soft palate (1), base of tongue (1). The AJCC/UICC stage ranged from Stage II to IVB. Two of the 4 patients received surgical intervention, with all 4 patients receiving CRT. Three of the patients were non-smokers, one a current smoker. Two patients consume alcohol, but not in excess. One patient had a history of prior consuming alcohol in excess, with the alcohol status of the final patient unknown. Three of the 4 patients did not have any identified LR and/or DM and were alive at the time of this study being conducted.

One patient with dual HPV genotype detected, HPV 35 and 16 developed LR and/or DM. This patient was a male, aged 41 years at time of diagnosis with a primary tonsillar SCC, with AJCC/UICC Stage IVB at diagnosis. He was a smoker at time of diagnosis, with alcohol consumption status unknown. The DFI was 5 months.

3.5.2 HPV 16 Lineage

HPV16 sub-lineage analysis was conducted as outlined in Section 2.5.4. Forty five of the 63 samples (71.43%) of samples aligned to European Prototype sub-lineage. Sixteen of the 63 samples (25.4%) aligned to the European German sub-lineage. One case (1.59%) aligned to the North American 1 sub-lineage and the Asian American 1 sub-lineage. The findings are outlined in the respective phylogenetic trees in *Figure 3.6.*

In relation to statistical analysis, a statistically significant correlation was not identified between the European Prototype sub-lineage and the recurrence group (n=61, odds ratio 0.6588235, p = 0.523, 95% C.I. 0.1833603 - 2.367189). A statistically significant correlation was also not identified between the European

Prototype sub-lineage and the non-recurrence group (n=19, odds ratio 0.333333, p = 0.376, 95% C.I. 0.0292411- 0.4470826). Statistical analysis was not conducted on the other HPV 16 sub-lineages detected due to the low numbers detected.

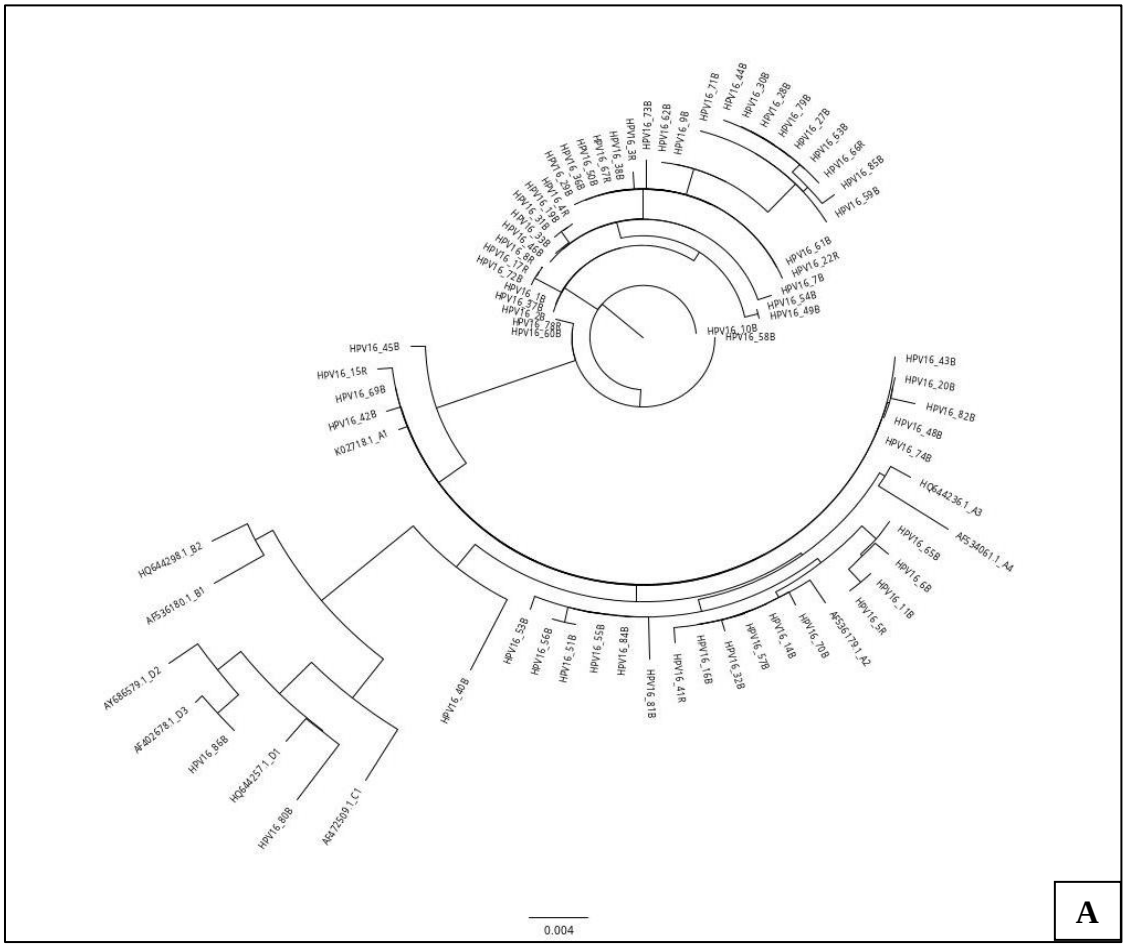

Figure 3.6 (A) *Phylogenetic tree representative of the HPV 16 sub-lineages identified.*

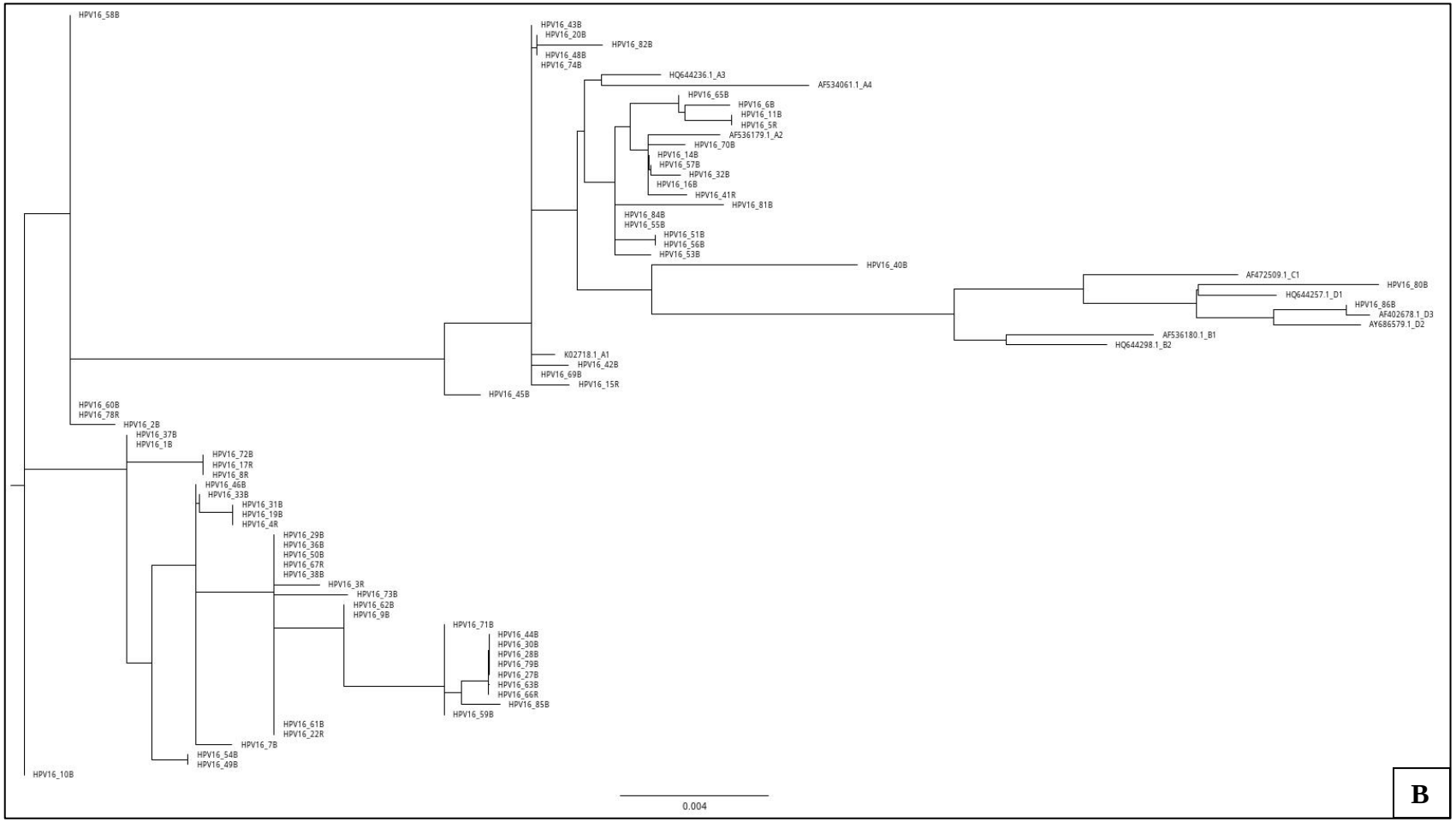

Figure 3.6 (B) *Phylogenetic tree showing representative HPV 16 sub-lineages.*

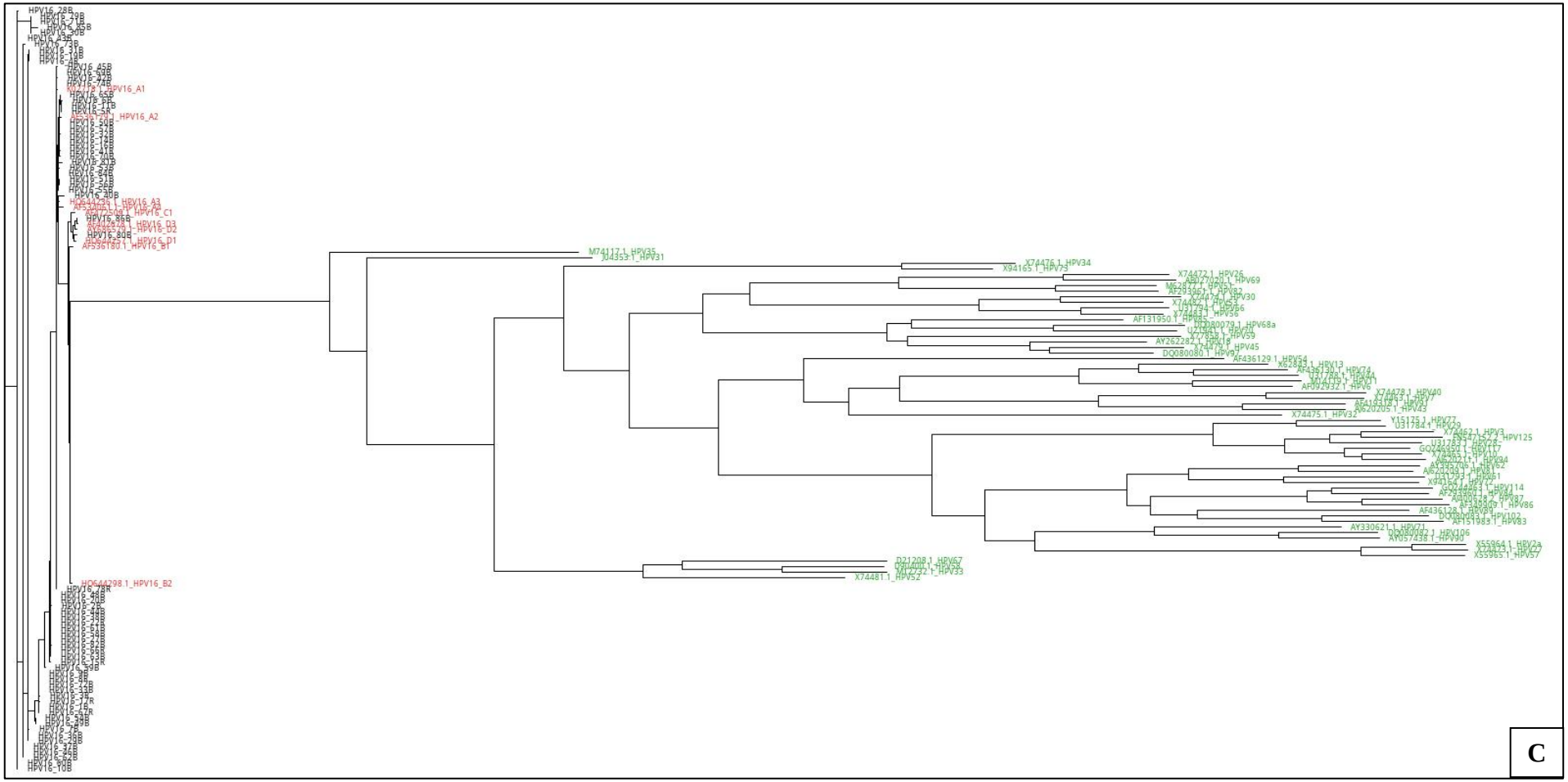

Figure 3.6 (C) *Phylogenetic tree showing representative HPV 16 sub-lineages. Non-HPV 16 subtypes illustrated in green, HPV16 sub-lineages illustrated in red and samples within this study illustrated in black.*

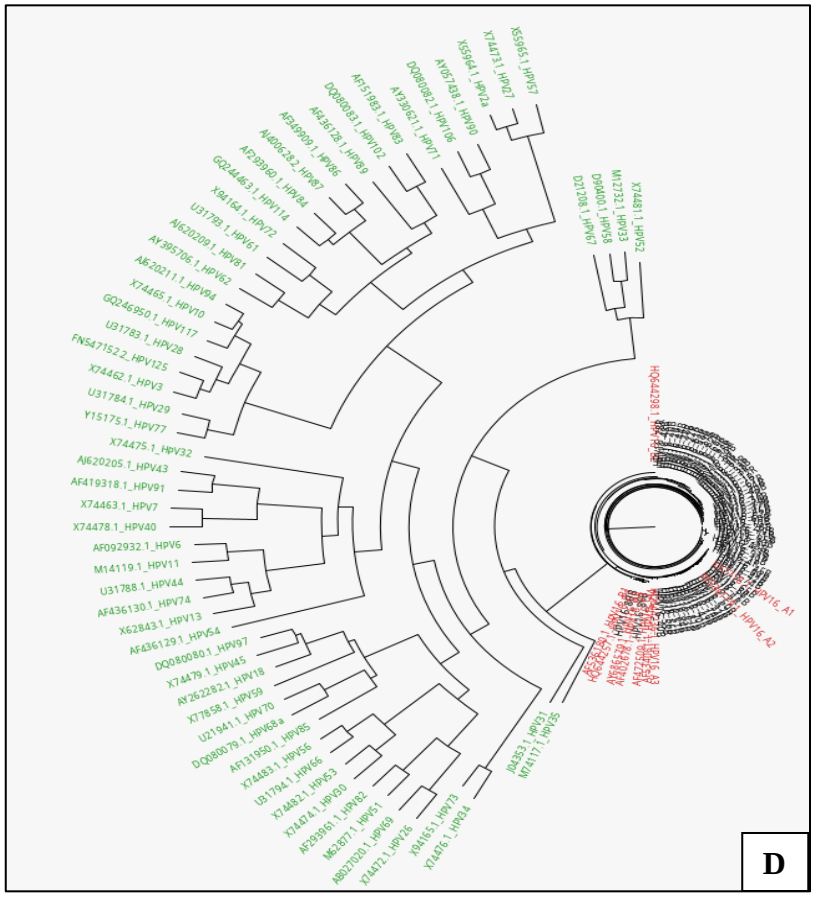

Figure 3.6 (D) *Phylogenetic tree showing representative HPV 16 sub-lineages. Non-HPV 16 subtypes illustrated in green, HPV16 sub-lineages illustrated in red and samples within this study illustrated in black.*

3.6 Somatic Mutation Detection via smMIP Panel Based NGS

59 samples had sufficient DNA concentration to conduct somatic mutation analysis on the study samples *(Figure 3.7).* Of the 59 samples, 11 failed the quality control standard metrics set. Eleven of the 59 (18.64 %) of the samples failed the QC standard set, with 48/59 (81.36 %) passing. Of note, 3 of the 11 samples who failed were from patients with LR and/or DM. Analysing the 48 who passed the QC standard, 3 (6.25 %) had a detectable mutation. The mutations outlined include BRAF, KRAS and NRAS, displayed in *Table 3.24.*

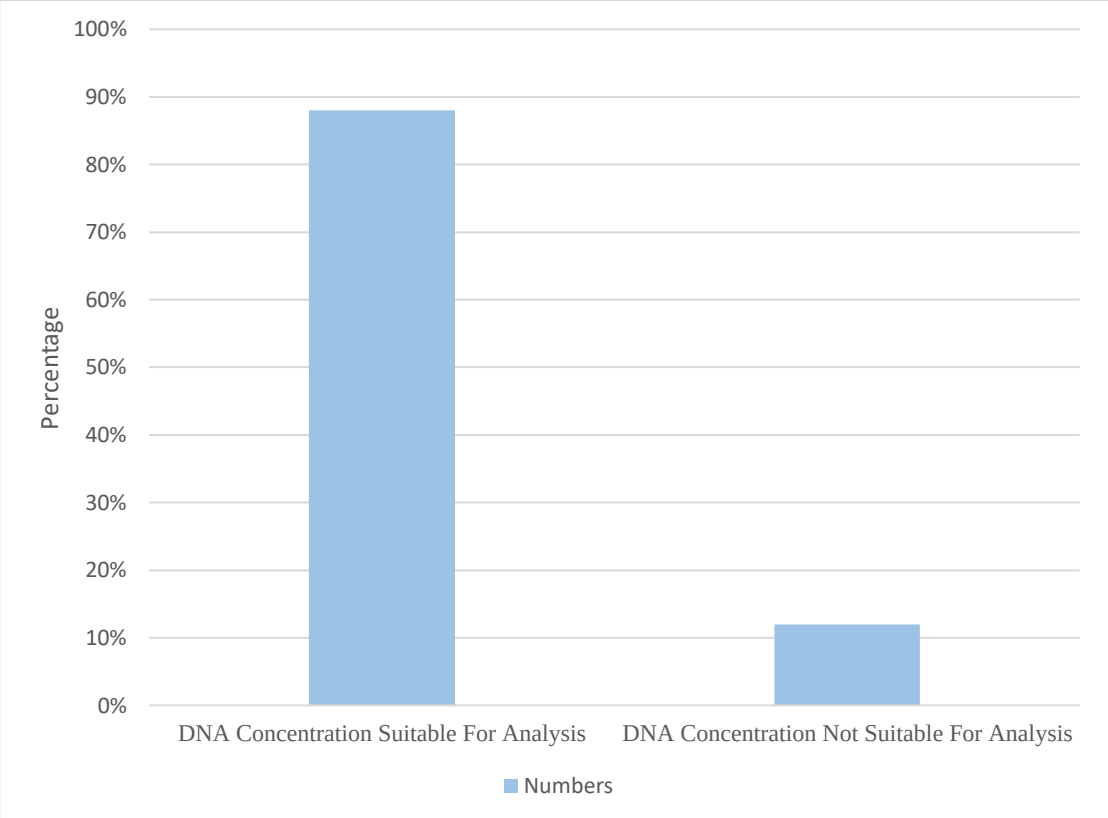

Figure 3.7 *Percentage of Samples Suitable for smMIP Panel Analysis Based on DNA Concentrations Achieved During DNA Extraction From FFPE. Suitable (n=59), Unsuitable (n=8).*

Somatic Mutations Identified via smMIP Panel based NGS				
	Chromosome	**cDNA**	**Protein**	**Percentage**
KRAS	**12**	**c.34G>A**	**p.G12A**	**7.5**
BRAF	**7**	**c.1781A>G**	**p.D594G**	**11**
NRAS	**1**	**c.182A>G**	**p.Q61R**	**15**

Table 3.24 *Somatic Mutations Identified via smMIP Panel Based NGS.*

The patient demographics and HPV genotypes identified of the patients with somatic mutations identified via smMIP panel based NGS is illustrated in *Table 3.25.* Of note, all patients were alive at the time of this study, with no cases of LR and/or DM or a new incidental primary identified

Somatic Mutations Identified via smMIP Panel Based NGS										
Mutation	**Gender**	**Age (years)**	**Subsite**	**AJCC/UICC Stage**	**Treatment**	**LR and/or DM**	**RIP**	**HPV Genotype**	**Smoking Status**	**Alcohol Consumption**
KRAS	**Male**	**57**	**Soft Palate**	**IVA**	**CRT**	**No**	**No**	**33, 16**	**Non-Smoker**	**Prior Excess**
BRAF	**Male**	**70**	**Tonsil**	**IVA**	**CRT**	**No**	**No**	**16**	**Current Smoker**	**Consumer, Not in Excess**
NRAS	**Female**	**56**	**Tonsil**	**IVA**	**Surgery and CRT**	**No**	**No**	**16**	**Non-Smoker**	**Non-Drinker**

Table 3.25 *Patient Demographics and HPV Genotypes Of the Patients with Detected Somatic Mutations. Abbreviations: CRT, Chemoradiotherapy*

3.7 Quality Control Measures

During this study, QC measures were utilised to provide assurance that attained results met the highest standards. QC standards were implemented for HPV genotyping and somatic mutation detection. *Figure 3.8* illustrates the breakdown of samples sourced for this study in relation to their performance against the QC standards.

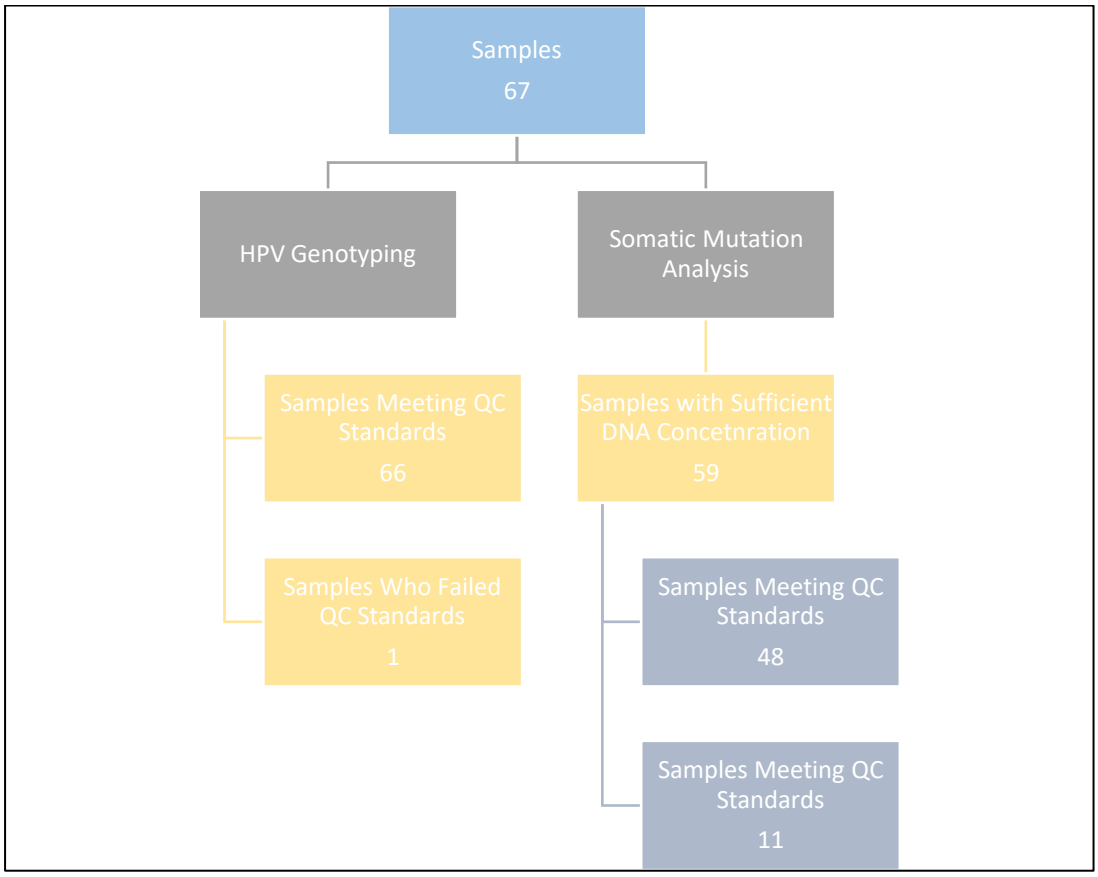

Figure 3.8 *Breakdown of Samples Sourced For This Study and Their Performance Against The QC Standards.*

Firstly examining the QC standards set for HPV genotype analysis via Ion Torrent NGS outlined in *Chapter 2*, a standard of an average of 100 reads from the human reference genes per samples was instituted. Reads were identified for the 4 human reference genes utilised for normalisation. One specimen, did not have any identified reads for RPL33 and RPS27A. However, BTF3 and UBA52 were detected with a read count of 5 for BFT3 and a read count of 2 for UBA52. Given the low read counts and thus failure to meet QC standards, this case was excluded from the study. The case that did not pass QC standards was not associated with LR and/or DM.

Secondly examining the QC standards set for somatic mutation analysis via smMIP panel based NGS outlined in *Chapter 2*, 11/59 (18.64 %) failed the QC standard set, with 48/59 (81.36 %) passing *(Figure 3.9)*.

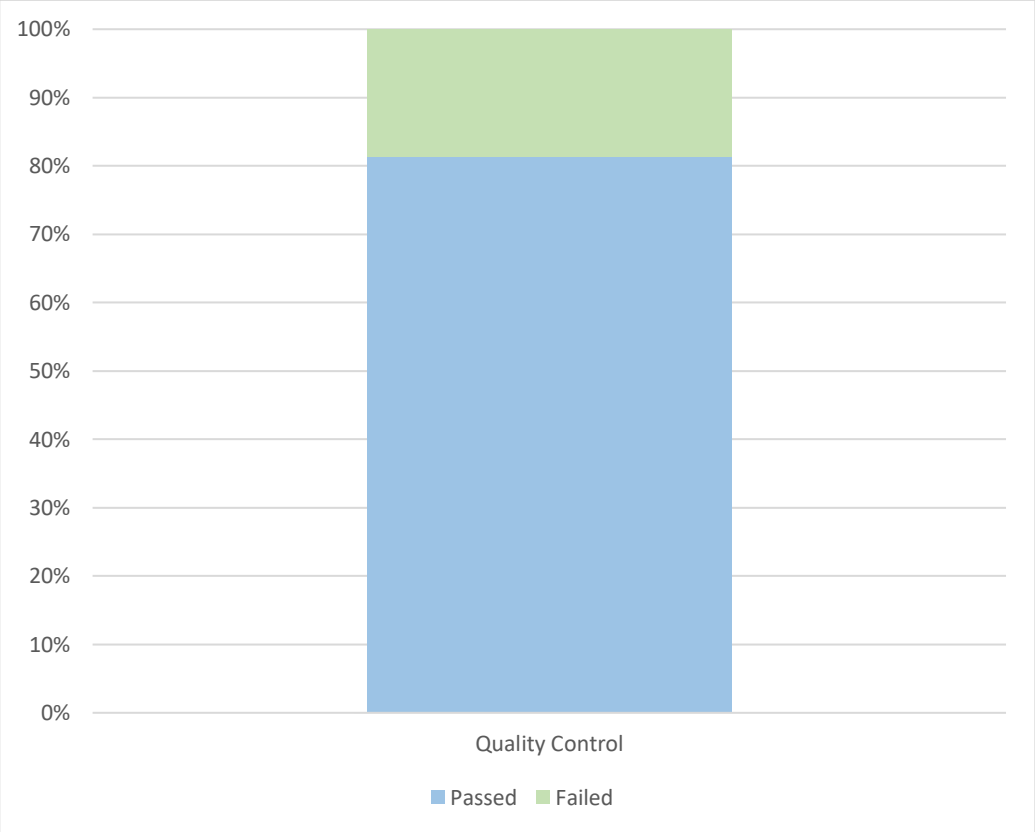

Figure 3.9 *Percentage of Samples That Passed The smMIP based Quality Control Measures. Pass (n=48), Fail (n=11).*

From the 11 samples, 9 (81.82%) were from DNA extracted from FFPE tumour tissue from a biopsy specimen; 2 (18.12%) were from DNA extracted from FFPE tumour tissue from a resection specimen. However, in contrast to the 48 samples who did meet the quality control standard set, 40 (83.33%) were from DNA extracted from FFPE tumour tissue from a biopsy specimen; 8 (16.66%) were from DNA extracted from FFPE tumour tissue from a resection specimen. The sample numbers within this study were insufficient to draw any conclusion addressing the correlation with a biopsy or resection specimen and achieving QC standards. Of note, there was no overlap of the sample who failed QC standard in HPV genotyping and the samples who failed the smMIP panel based NGS QC standards.

Scrutinising the samples who did not achieve the QC standards, 10/11 (90.9 %) were from historic FFPE tumour tissue blocks prior to 31/12/2012. In total, from the 27 samples from the 01/01/2007 to 31/12/2012, 10 failed the quality control standards (37.04 %). The somatic mutation analysis investigated for this study was conducted in August 2022, thus DNA was extracted from samples that were a minimum 9.6 years old.

From the samples from 01/01/2013 to 31/12/2016, 1 (9.1 %) failed the QC standards. In total, from the 32 samples from the 01/01/2013 to 31/12/2016, 1 failed the quality control standards (3.13 %).

Chapter 4

Discussion

HPV is a known causative factor leading to the development of OPSCC, with numbers attributable to the virus increasing [9, 14, 15]. As HPV positive OPSCCs are identified in a younger cohort of patients and are associated with a more favourable prognosis in contrast to HPV negative OPSCC, the implications of surgical excision, chemotherapy and/or RT may result in long standing disabling effects on an individual's quality of life [44, 45].

Despite the advantageous prognosis proffered, it is approximated that 10 – 15% of patients with HPV positive OPSCC will develop LR and/or DM [239]. This study sought to establish if there are particular HPV genotypic strains and/or specified somatic mutations linked with the development of LR and/or DM in HPV positive OPSCC.

In addition, this study aimed to establish if a correlation was present between patient demographics and the identified HPV genotype(s) and/or specified targeted mutation.

The final aim of this study was to illustrate if an achievable pathway could be instituted to conduct genetic testing on FFPE tumour tissue for use in prospective samples, in the event that genetic aberrations were identified.

Armoured with such information, this study endeavoured to address if particular tumour biological factors could be ascertained to assist in future de-intensifying studies/strategies to minimise challenging long term side effects experienced by patients based on current therapeutic regimens.

4.1 Correlation Between Patient Demographics and HPV Positive OPSCC With Locoregional Recurrence and/or Distant Metastasis

LR and/or DM was identified in 19 (28.36 %) of patients enlisted in this study (30.16 %, excluding those for whom adequate follow-up had not been sourced). The overall rate of LR and DM was 19.05 % and 11.11 %, respectively.

Limited studies exist examining LR and/or DM in HPV positive OPSCC, with LR observed in 13.22 % of cases with a mean follow up of 3.7 years, and a rate of DM of 9.47 % with a mean follow up of 3.56 years [162, 240-243]. Characteristically, cases of recurrence occur within the first two years post primary treatment [243, 244]. Thus, the higher rates of LR and DM witnessed in this study may not be attributable to the longer follow up period. The aetiology of higher recurrence rates within this study has not been elucidated.

Age

The mean age at diagnosis of 57.26 years is in keeping with the findings from cited literature [44, 45, 53, 245, 246]. However, the age profile of individuals diagnosed with HPV positive OPSCC has increased over the preceding decades [239, 246-249], which was also found in this study, albeit to a lesser extent.

The average age at time of diagnosis with HPV positive OPSCC for those who subsequently developed LR and/or DM was 54.16 years. Of interest, the age of diagnosis for those who did not develop LR and/or DM was 58.16 years. The younger the age at diagnosis correlated with an increased likelihood of LR and/or DM ($p = 0.057$).

This finding is unique to this study. Limited research to date suggests that cases of recurrence classically occur at a later age in contrast to HPV negative OPSCC [250]. In addition, an older age at diagnosis with HPV positive OPSCC has been characterised as lacking a survival benefit in contrast to HPV negative disease [247, 251]. Of note, this study did not review patients with HPV negative OPSCC.

Considering this observation, future studies are warranted to assess if a younger age at diagnosis may be suggestive of poorer outcomes for patients with HPV positive OPSCC. Such findings may have negative connotations for current de-intensification strategies and is thus a pivotal finding from this study which requires future research.

American Joint Committee on Cancer/ Union for International Cancer Control Stage

This study established a statistically significant correlation between a higher clinical stage of primary disease and LR and/or DM when age, gender, smoking status, and alcohol consumption were accounted for (n=50, odds ratio 6.026, p = 0.01, 95 % C.I. 1.52513 – 23.8102). These findings corroborate previously published studies [162, 252, 253].

Lifestyle Factors: Smoking and Alcohol

The stated prevalence of individuals who are current or former smokers at time of diagnosis of HPV OPSCC ranges from 36 % to 54 % [254-257]. However in this

Irish cohort, 73.1 % of patients were either active or former smokers at the time of diagnosis.

Additionally, this study highlighted that 18/19 (94.74 %) patients who developed LR and/or DM were current or former smokers at the time of diagnosis. This figure contrasts with 30/42 (68.18 %) in the non-recurrence group. Limiting the field to those who developed a LR, 12/12 (100 %) were current or formers smokers at time of diagnosis. However, the results showed no statistically significant correlation between smoking status, whether current or prior, with the development of LR and/or DM ($p = 0.189$).

Nevertheless, the effects of smoking have been shown to play a significant impact on patient outcomes in terms of OS and DFS in HPV positive OPSCC [162, 254, 258-260]. One could hypothesise that smoking would be associated with an increased risk of disease recurrence, primarily LR, as smoking would be a direct insult in this region. However, research to date has highlighted opposing results.

A systematic review by Chen *et al.* illustrated that 'heavier smokers' had 0.55 to 5.2 times increased hazard of LR in contrast to 'lighter smokers' [258]. However, isolated studies highlighted no correlation [261]. As the OS and DFS are reduced in active smokers, this poses the question as to whether smokers should be excluded in de-escalation treatment measures in future studies [254].

Concerning alcohol consumption, 74.63 % of patients were consuming alcohol at the time of diagnosis. The rates within published literature cover a wide range, 33 % to 90 % [239, 257, 262-265]. Forty percent of the study cohort consumed alcohol in excess at the time of diagnosis. In addition, 4 (80 %) of former alcohol consumers, met the criteria of excessive alcohol consumption. There is limited data

extrapolating such factors, with published figures ranging from 37 % to 68 % [262, 264, 266].

A statistically significant correlation was identified between current or prior excessive alcohol consumption at the time of diagnosis and the development of LR and/or DM when age, gender and smoking status were accounted for. In addition, a statistically significant correlation was identified between the DFI and current or prior excessive alcohol consumption when the exact HPV genotype is known (n=15, coef. -20.323, p = 0.033, 95 % C.I. -36648 – -1.998).

This is the first documented study within the literature to show a correlation of excessive alcohol consumption with LR and/or DM. These findings illustrate that further research is required to accurately define the role alcohol consumption confers with the development of LR and/or DM in HPV related OPSCC.

4.2 Correlation Between HPV Genotype and HPV Positive OPSCC With Locoregional Recurrence and/or Distant Metastasis

Cases of HPV 16 driven OPSCC accounted for 93.65 % within this study, with the prevalence determined from the literature ranging from 70 to 90% of cases [110, 119-123]. Cases of HPV 18 driven OPSCC were not identified within this study, with the prevalence determined from the literature of HPV 18 within the oropharynx ranging from 0.6 % to 4 % [110, 112, 125-127]. However, HPV 18 is cited as more prevalent within the black population [267]. Within this study, all the

patients were Caucasian, of Irish descent. Thus, a lower prevalence of HPV 18 would be expected.

This study identified four cases exhibiting dual HPV genotype strain. Co-infection is documented within the literature with limited numbers similar to this study, corroborating findings [110, 127].

The correlation between HPV genotypes with disease progression is unsubstantial and opposing [137, 142, 196-198]. As HPV 16 is the dominant genotype identified in literature, the numbers ascribed to the other HR genotypes is limited. Moreover, there are no focused studies investigating whether dual or multiple HPV genotypes are shown to correlate with disease progression or survival.

Consequently, dedicated research examining larger cohorts of patients, focusing on non - HPV 16 genotypes, and the impact of dual or multiple genotypes in the oropharynx may be a path worth investigating in future studies.

4.3 Correlation Between Somatic Mutations and HPV Positive OPSCC With Locoregional Recurrence and/or Distant Metastasis

Within this study, 3 individual somatic mutations were identified: KRAS, NRAS, BRAF. None of the identified cases were found in patients with LR and/or DM.

The RAS protein plays a pivotal role in cell signalling pathways, motility and survival [123, 268]. A recent meta-analysis and systematic review of RAS mutations in head and neck cancer showed that HRAS was the most frequently

mutated RAS family member (6.63 %), with similar values established for KRAS (2.89 %) and NRAS (2.2 %) [269].

Further in-depth scrutiny of tumour site within the head and neck showed that HRAS was the most prevalent RAS family member mutated within the oropharynx (2.6 %), followed by KRAS (1.49 %) and NRAS (0.65 %). Filtering the results from the current literature illustrates the prevalence of KRAS mutations in the HPV positive HNSCC cohort at 10 %, highlighted in *Table 1.2* [137, 140-154].

Pertaining to NRAS within a HPV positive HNSCC cohort, figures average at 2.35 % [200, 201, 219, 270-272]. Of note, these findings are restricted to the head and neck, and not directly centred upon the oropharynx due to the limited data available to date on this topic.

BRAF (v- rapidly accelerated fibrosarcoma murine sarcoma viral oncogene homolog B1) is a serine/threonine protein kinase involved in the RAS-RAF-MEK-ERK MAPK cell signalling pathway [273, 274]. Activation of this pathway leads to altered gene expression and continual progression through the cell cycle, unlimited growth and resultant neoplastic proliferation [273, 274]. Within this study, one case of BRAF V600E mutation (2.08 %) was identified.

The prevalence of BRAF mutations in HNSCC range from 0 % to 3 % [275-280]. There are no further studies narrowing the field concerning avidity to a specific site within the head and neck, or its relationship to HPV status. In the era of personalised medicine, BRAF inhibitors play a vital role in treatment of melanoma [281]. Further research is warranted to assess if such molecular tumour testing may be of benefit in patients with HNSCC/OPSCC.

4.4 Utilisation of smMIP Panel Based Next Generation Sequencing on FFPE Tumour Tissue

Recent advances in personalised medicine and the advent of targeted therapies for laboratory detected oncogenic mutations have led to greater treatment options for cancer patients. However, such advances have resulted in exponential challenges for pathology departments. Detection of targeted somatic mutations via smMIP panel based NGS is a novel, low cost, intuitive platform implemented to circumnavigate this issue. The smMIP panel used within this study was designed in CMD Laboratory, St James's Hospital, which is used to detect variants in both solid tumours and myeloproliferative neoplasms.

Data ara limited with respect to the application of smMIP panel based NGS for HNSCCs. A single study adopted smMIP panel based NGS for HPV positive and negative HNSCC derived from cell lines solely for research purposes [282]. The application of smMIP has been conducted on head and neck tumours including SCCs from frozen biopsy samples with low tumour cellularity, showing a sensitivity value of >95 % [283]. Additionally, the comprehensive list of somatic mutations targeted in this study has been well documented on FFPE solid tumour tissue outside of HNSCC [236, 284].

This study utilised archival FFPE tumour tissue. However, the genomic DNA extracted from FFPE tissue specimens is considered of lower quality [285]. This can result in inaccurate or failed genomic analysis. This study assessed measures ascribing to failures in targeted somatic mutation analysis and illustrated potential pitfalls to reduce future failures for this technique.

Tissue age can be an issue. From the literature smMIP panel based NGS was usually performed on DNA samples extracted were from samples up to 5 years old [236-238, 286-288]. One study had a far greater period of historic archival tissue ranging over a period of 11 years [238]. Regarding the age of the specimens within this study, 37.04 % of samples from pre 01/01/2013 failed the QC. On average samples were at a minimum 9.5 years old. Owing to the small sample number, statistical analysis was not feasible. However, historic tissue may not be amenable for retrospective analysis.

In this study, DNA was extracted from FFPE tissue scrolls. Tumour cellularity was reviewed on the H&E prior to scrolls taken. However, the tumour cellularity may reduce drastically, particularly in biopsy specimens, when cutting in at time of microtomy. Furthermore, extracted DNA would have included background non-tumour cells, possibly minimising the true tumour cellularity. Alternate methods of extraction not explored in this study include macro or micro - dissection. These factors are all variables to consider outside of the age of the FFPE samples.

Concluding findings from this study show that FFPE tumour tissue can be effectively utilised as a DNA source for targeted somatic mutations in OPSCC. Due to the small sample number, further verification and validation studies are warranted however.

4.5 Study limitations

This study is not without its limitations. The central limitation is the cohort size. As HPV driven tumorigenesis within the oropharynx may be deemed a more recent

discovery, historic cases as limited. This is highlighted by an Irish 10-year retrospective study which highlighted only 86 HPV positive OPSCC cases [289]. This results from this study were centred on patients from the SJH HNC database. SJH is the national Head and Neck Cancer Centre, and thus would be expected to provide a comprehensive reflection of national figures. Data from 2020 states that 98 cases of HPV driven OPSCC are diagnosed in Ireland annually [290]. Thus, future studies with prospective cases may yield greater cohort sizes.

An additional limitation pertains to the variability of the data collected. This was a retrospective cohort study, with reliance on historical documentation within each patient's medical chart, SJH EPR and the LIS. Historical data collection was incomplete for the following parameters examined in this study: smoking status, alcohol consumption, TNM staging, AJCC/UICC clinical staging, treatment regimen received and recurrence status. With respect to cases for which data was not available, the relevant parameter was designated a status of 'unknown'. Incorporating missing historical data with the small study size limited the statistical power of the study.

A further limitation related to the volume of invasive carcinoma present within the FFPE tumour blocks. The FFPE tumour specimen blocks were predominantly composed of diagnostic biopsies, from which the sample size taken was dependent on the judgement by the operative Head and Neck Surgeon. Furthermore, despite all cases having confirmatory invasive SCCs, the ratio of cancer to background benign tissue was low in certain samples analysed on the H&E slide examined under light microscopy. In addition, extra unstained sections would have been taken at microtomy to conduct p16 IHC staining as a marker for HPV status, contributing to reduced amount of tumour tissue. These factors are likely to have contributed to the

reduced DNA concentration, which limited the number of samples that could be analysed.

An additional limitation related to the selected smMIP panel that was readily available. A broader mutation panel may have yielded additional information.
A potential limitation pertains to the tissue sampled from which somatic mutation analysis was conducted. Testing for genetic aberrations was conducted on FFPE tumour tissue from the primary diagnostic histological specimen and not on a histological sample taken from the recurrence and/or metastasis site in line with study aims. However, analysis of garnered mutations in the LR or DM tumour may have provided additional information that may have influenced survival outcomes.

4.6 Study Applications

This study has identified a relationship between a number of patient demographic factors such as current or prior excessive alcohol consumption, higher clinical stage of primary tumour, and lower age at diagnosis, with the development of LR and/or DM in HPV positive OPSCC. With ongoing developments in de-escalation measures to reduce morbidity for patients with HPV positive OPSCC, careful vetting criteria will be needed to ensure appropriate patient selection for clinical trials.
Furthermore, this study has illustrated the feasibility of utilising archival FFPE tumour DNA for somatic mutations detection via smMIP Panel Based NGS.

4.7 Conclusions and Recommendations for Further Research

In the era of personalised medicine, it is hoped that interrogation of archival FFPE tumour tissue via similar techniques used in this study may yield pioneering findings with a clinical and prognostic significance. Future directives including addressing the viability of historic FFPE tumour tissue on a larger cohort of samples, may provide key laboratory findings which may direct treatment regimens.

In conclusion, it is hoped that this research will contribute to the growing body of knowledge and inspire further research into the area of LR and/or DM in HPV positive OPSCC.

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Appendices

Appendix 1: Consent Documentation

Appendix 1.1: Invitation Letter – SJH

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

ST. JAMES'S HOSPITAL

To whom it may concern,

You are invited to be part of a study examining head and neck cancer. The study will be examining tumour tissue stored within the hospital and a review of hospital medical records.
Participants will not be required to attend the hospital at any point or to provide any further samples or information.
Please find enclosed a detailed patient information leaflet, summary and consent form.
If you are happy to be part of this study, please return a signed consent form in the envelope provided.
If you have any issues please contact me on 01-8963289.

Kind regards,

Dr Shane Brennan
Histopathology Specialist Registrar
MB Bch BAO MRCPI Dip RCPath CHAT
Co-Investigator
Trinity College Dublin

31/03/2021

Appendix 1.2: Patient Information Leaflet - SJH

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

ST. JAMES'S HOSPITAL

Project:
Human Papillomavirus associated Oropharyngeal Squamous Cell Carcinoma:
Genetic Aberrations Associated with Local and Distant Recurrence.

Principal Investigator:

Professor Orla Sheils,
Dean of Health Sciences, Trinity College Dublin.

Co-Investigators Investigators:

(i). *Dr Esther O'Regan*, Consultant Histopathologist, St. James's Hospital, Dublin.
(ii). *Dr Shane Brennan*, Histopathology Specialist Registrar, St. James's Hospital, Dublin.
(iii). *Dr Anne-Marie Baird*, Research Fellow, Trinity Translational Medicine Institute, Trinity College, Dublin.
(iv). *Professor Susan Kennedy*, Consultant Histopathologist, Royal Victoria Eye and Ear Hospital, Dublin.
(v). *Dr Stephen Crowther*, Consultant Histopathologist, Tallaght University Hospital, Dublin.
(vi). *Mr Paul Lennon, Consultant Head and Neck Surgeon, St. James's Hospital, Dublin*

Date: 14/06/2020

Introduction:

You are being invited to take part in a research study, which is being conducted in the Trinity Translational Medicine Institute, St. James's Hospital, Dublin. Before you decide to participate, it is important for you to understand why the research is being done and what it will involve.

Please take time to read the following information and discuss it with family, friends or others if you so wish. Please contact any member of our investigative team if there is anything that is not clear or if you would like more information.

Thank you for reading this.

Part One: The Study

Why is this study being done?

The oropharynx is a segment of the autodigestive tract. The most common cancer type in this site is a type of cancer referred to as squamous cell cancer. For cancers of this site, the Human Papillomavirus (HPV) is a known risk factor.

The study is being conducted to identify which particular strains of the Human Papillomavirus are present in the cases in our institution. Furthermore, the study is being conducted to identify particular mutations in the tumour DNA of patients with this cancer type.

We hope that this study will further our knowledge as to why cancers of the head and neck area occur and help to guide the best treatment regimens for patients.

Research aim(s):

The aim of this research is to:

1. ***Identify particular strains of the Human Papillomavirus in the tumour.***
2. ***Study how often certain mutations within the tumour are seen.***
3. ***Assess if there is a link between these two factors and disease progression.***

Why am I being asked to take part?

Participants in this study have been diagnosed with cancers of the head and neck in the region referred to as the oropharynx from 01/01/2007 to 31/12/2016.

Do I have to take part? What happens if I say no? Can I withdraw?

Your participation in this research project is voluntary. You are not obliged to partake. Should you decide not to partake, there will be ***no*** adverse effects to you, your treatment or your future care. Should you decide to withdraw, you are ***not*** required to provide a reason for your decision.

Please note, should you wish to withdraw, data collected up to the point of your withdrawal will be kept and used in the study.

Please also note that there will be a certain point at which you will not be able to withdraw from the study.

Once all data has been collected, Dr Brennan will irreversibly anonymise the data.. When data is irreversibly anonymised, it will not be possible for investigators to identify which data relates to you. At this point, it will not be possible to withdraw from the study

If you wish to do withdraw prior to data being irreversibly anonymised, please contact Dr Shane Brennan in St. James's Hospital on 01-8963289 between the hours of 09:00 to 17:00 Monday to Friday, and your participation in the project will be stopped.

How will the study be carried out?

The study you are being asked to partake in will take place in the Trinity Translational Medicine Institute, St. James's Hospital, Dublin 8.

The study will start in January 2020 and continue over a 2-year period.

In total, we are aiming to recruit 100 patients to be involved in this study.

The study will be carried out on your tumour tissue already archived in the Histopathology Laboratory of St. James's Hospital or Tallaght University Hospital at the time at which you had your tumour biopsy or resection.

This material will then be processed in the laboratory to look for the strain of the Human Papillomavirus and to look for specific targeted mutations within the tumour DNA.

What will happen to me if I agree to take part?

This study will not require you to attend.
The study will not require you to provide a sample.
This study will not require you to undergo any follow up.

No further personal information, material, testing or samples will be required.

There will be no medical or surgical interventions.

There will be no change to your treatment regimen if you are on one.

If you agree to take part, you will be providing investigators access to personal data that is listed in Part 2.

If you agree to take part, you will also be providing investigators access to your tumour tissue already stored from the time at which you were diagnosed with cancer.

When you had your biopsy or tumour resection, the tumour tissue was preserved with a special agent and then made into a wax block and stored. The study involves accessing the stored tissue in the laboratory. A tiny portion of the tissue in the block is utilised to extract DNA material.

This material will then be processed in the laboratory to look for the strain of the Human Papillomavirus and to look for specific mutations with in the tumour DNA.

Are there any benefits to me or others if I take part in the study?

The study may have no direct benefit to you but it is hoped that the results may benefit subsequent patients with similar cancers in the future.

Are there any risks to me or others if I take part in the study?

No.

As this study does not involve any medical or surgical intervention, or change to a treatment regimen, this study does not put you at risk.

Will I be told the outcome of the study? Will I be told the results of any tests or investigations performed as part of this study that relate to me?

You will not be informed of the outcome of the study. If you would like to be informed of the overall results of the study, the final research report can be made readily available. Please note you will not be able to identify your data from the results as data from all participants will be combined. Note that any outcome from this research will not directly or indirectly impact on your health.

Should this study provide results that may be of national or international interest, the findings may be shared via medical journals or medical conferences. If so, the results from the study are completely anonymised. There will be no possible means by which your case could be tracked/made identifiable.

Part Two: Data Protection

What information about me (personal data) will be used as part of this study? Will my medical records be accessed?

If you agree to partake in this study, you will be providing investigators access to:

1. Your Medical Chart, which will be accessed by the Ear, Nose and Throat Surgical Department.
2. Your record on the Electronic Patient Record in St. James's Hospital
3. Your histopathological report on the Laboratory Information System in the Histopathology Laboratory in St. James's Hospital and/or Tallaght University Hospital.

From this, data we are looking to ascertain includes:

1. Age
2. Gender
3. Date of Diagnosis
4. Time period between initial diagnosis and recurrence
5. Treatment regimen received
6. Smoking status
7. Alcohol consumption
8. HPV status determined at time of diagnosis

Data collected initially will be pseudonymised. Once all data above is collected the data will be irreversibly anonymised, which means that data cannot be traced back to you. Your case will be allocated a code and thus no identifiers will be available to link back the case to you. At this point it will not be possible to be removed from the study.

What will happen my personal data? Will my personal data be kept confidential? How will my data be kept safe?

It is our priority that any data provided by participants is kept safe and secure.

Only data points listed above will be reviewed by our investigative team. Your personal data will be processed only as is necessary to achieve the objective of the health research and will not be processed in a way that damage or distress will be caused to you.

Data obtained will be stored in the Sir Patrick Dun Research Laboratory of the Trinity Translational Medicine Institute and the Central Pathology Laboratory, St. James's Hospital.

The next steps outline the mechanisms in place to ensure the security of your data:

Firstly, in order to enter the Sir Patrick Dun Research Laboratory and the Central Pathology Laboratory, St. James's Hospital, swipe access is required. There are members of security at the entrance to the building.

The returned consent forms will be stored in a locked filing cabinet in a locked room in the Sir Patrick Dun Research Laboratory, St. James's Hospital.

For those participants diagnosed in Tallaght University Hospital, coded pseudonymised data will be stored in a Microsoft Excel spreadsheet, on a password protected, encrytped computer in Tallaght University Hospital Pathology Laboratory.

The Microsoft Excel spreadsheet will then be transferred securely to a St. James's Hospital email address of Dr Shane Brennan. Once that has occurred, the Microsoft Excel spreadsheet in Tallaght University Hospital will be deleted.

For those patients diagnosed in St. James's Hospital, coded pseudonymised data will be stored in a Microsoft Excel spreadsheet, on an encrytped computer in St. James's Hospital Pathology Laboratory.

The pseudonymised data from the Tallaght University Hospital Microsoft Excel spreadsheet securely sent to Dr Brennan's St. James's Hospital email will be opened on an encrytped computer in St. James's Hospital Pathology Laboratory.

The Tallaght University Hospital and St. James's Hospital Microsoft Excel spread sheets will be combined on a password protected, encrypted computer in St. James's Hospital Pathology Laboratory. Once all the data is collected it will be irreversibly anonymised.

Once this has occurred, this irreversibly annonymised data will be analysed on a Microsoft Excel spreadsheet on a secure server in the Sir Patrick Dun Research laboratory, Trinity Translational Medicine Institute, on St James's Hospital Campus.

Only irreversibly annonymised data will be accessible to the other study investigators. An assessment of the data protection implications of the health research and /or a data protection impact assessment was carried out and no high risk (either in terms of likelihood or severity) issues to personal data breach to the rights and freedoms of the data subject were identified.

Data will not leave the St. James's Hospital/Trinity College Dublin Campus.

Data will not be leaving the State.

Data will be stored for a maximum of 5 years, after which time all data will be deleted by Dr Shane Brennan, Histopathology Specialist Registrar and Co-Investigator on this project. A period of 5 years is given to ensure that the research project is completed within that period. Should the project be completed prior, data will be deleted at an earlier stage. Data will not be stored for any longer than is required for completion of the project.

Who will access and use my personal data as part of this study?

Your data will be accessed only by the investigators outlined on the first page of this document.

What is the lawful basis to use my personal data?

In relation to the Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC (General Data Protection Regulation), the lawful bases for processing as set out in Article 6 falls under category (e).
Processing is necessary for the performance of a task carried out in the public interest or in the exercise of official authority vested in the controller;
In relation to the processing of special categories of personal data under article 9, this research falls under category 2. (j).

processing is necessary for archiving purposes in the public interest, scientific or historical research purposes or statistical purposes in accordance with Article 89(1) based on Union or Member State law which shall be proportionate to the aim pursued, respect the essence of the right to data protection and provide for suitable and specific measures to safeguard the fundamental rights and the interests of the data subject.

What are my rights?

Rights held by all participants in this study are in keeping with those stated Rights of Individuals under General Data Protection Regulation. Each participant has the:

- Right to decline invitation to partake in the study
- Right to terminate participation in the study
- Right to access his/her data held
- Right to restrict the use of the data held
- Right to correct inaccuracies
- Right to have information deleted
- Right to data portability

- Right to object to profiling

Part Three: Cost, Funding, Approval & Future Research

Will it cost me anything if I agree to take part?

This study has no cost implications for you.

Who is funding this study? Will the results study be used for commercial purposes?

Professor Sheils will cover the costs of the consumables from established research funding.
€10,000 has been awarded for the research project from the Technicon Research Bursary. A further application for funding from the Pathological Society of Great Britain and Ireland has also been requested.

Sources of funding may be also sourced from other institutions should research grants become available. Note however, that funders will not be able to identify you within this study and will not be permitted to utilise data sourced.

Note there will be no funding or sponsorships from pharmaceutical companies. The researchers/investigators in this project will not be paid for their work on this project.

The results of this study will not be disclosed for commercial purposes.

Has this study been approved by a research ethics committee?

This study was approved by the joint

St. James's Hospital/ Tallaght University Hospital Joint Research Ethics Committee on the 24/02/20201.

The study has also been approved by the Royal Victoria Eye and Ear Hospital Research Ethics Committee.

Provide details of the **research ethics committee** that gave ethical approval to the research including:

- The **name and contact details** of the committee that gave ethical approval to the research (does not need to be a named individual).
- Whether any of the persons carrying out the research have **a link** to the committee or the institution behind the committee.
- The **date** ethical approval was given by the committee.
- **Reporting arrangements** agreed with the committee.

- Any **conditions** attached to the research by the committee.

Professor Orla Sheils is a member of the St. James's Hospital/ Tallaght University Hospital Joint Research Ethics Committee. Given her involvement, at time at which the research was put before the ethics committee, Prof. Sheils was not present to ensure no conflicts of interest.

Will my personal data and/or biological material be used in future studies?

No. Your personal data and/or biological material will not be used in future studies.

Part Four: Further Information

Where can I get further information?

For further information please contact:

- Principal Investigator:
  Professor Orla Sheils, Dean of Health Sciences; Professor of Molecular Diagnostics, Department of Histopathology; Director of Medical Ethics, School of Medicine; Chair of Research Ethics Policy Committee, Trinity College Dublin

  Tel: 01-8963284

- Data Controller:
  St. James's Hospital, Tallaght University Hospital, Royal Victoria Eye and Ear Hospital and Trinity College Dublin.

Alternatively, please contact:

Co-Investigator and Data Processor:
Dr Shane Brennan
Email: shbrennan@stjames.ie
Telephone: 01-8963289

What happens if I wish to make a complaint?

Should any issues arise, and you wish to contact a member of the investigative team to make a complaint, please contact the co-investigator Dr Shane Brennan on 01-8963289 in the Histopathology Laboratory of St. James's Hospital.

Should you have an issue regarding your data, you may wish to contact the Data Protection Officer of Trinity College Dublin/St. James's Hospital/Tallaght University Hospital or the Data Protection Commissioner's Office.

Contact details for the Data Protection Officers in St. James's Hospital, Tallaght University Hospital, the Royal Victoria Eye and Ear Hospital and in Trinity College Dublin are as follows:

1. Trinity College Dublin:
   Data Protection Officer,

   Secretary's Office,
   Trinity College Dublin,
   Dublin 2,
   Ireland.

   dataprotectionofficer@tcd.ie

2. St. James's Hospital:
   Data Protection Officer,
   St James's Hospital,
   James's Street,
   Dublin 8,
   Ireland.

   dataprotectionofficer@stjames.ie

   (01) 416 2463

3. St. Tallaght University Hospital:
   Data Protection Officer,
   St James's Hospital,
   James's Street,
   Dublin 8,
   Ireland.

   dpo@tuh.ie

   (01) 4142015

4. The Royal Victoria Eye and Ear Hospital
   Data Protection Officer,
   Royal Victoria Eye & Ear Hospital,
   Adelaide Road,
   Dublin 2,
   D02 XK51

   dpo@rveeh.ie

   (01) 708 8553

Will I be contacted again?

This is an open invitation to participate in this study. Should you wish to participate please reply with the self-addressed envelope.

Non-responders will be followed-up by a reminder letter and a telephone call.

However, should you not wish to participate, you may contact Dr. Shane Brennan on 01-8963289, or reply to this letter stating you do not wish to participate.

Appendix 1.3: Informed Consent Document - SJH

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

ST. JAMES'S HOSPITAL

INFORMED CONSENT DOCUMENT

Project: **Human Papillomavirus associated Oropharyngeal Squamous Cell Carcinoma: Genetic Aberrations Associated with Local and Distant Recurrence.**

Principal Investigators:

(i). Professor Orla Sheils, Dean of Health Sciences, Trinity College Dublin.
(ii). Dr Esther O'Regan, Consultant Histopathologist, St. James's Hospital, Dublin.
(iii). Professor Susan Kennedy, Consultant Histopathologist, Royal Victoria Eye and Ear Hospital, Dublin.
(iv). Mr Paul Lennon, Consultant Head and Neck Surgeon, St. James's Hospital, Dublin.
(v). Dr Stephen Crowther, Consultant Histopathologist, Tallaght University Hospital, Dublin.
(vi). Dr Shane Brennan, Histopathology Specialist Registrar, St. James's Hospital, Dublin.
(vii). Dr Anne-Marie Baird, Research Fellow, Trinity Translational Medicine Institute, Trinity College Dublin.

To be completed by the PARTICIPANT:

I have read and understood the patient information leaflet provided.	*YES* ☐	*NO* ☐	*Initials:*
I have had the opportunity to discuss the study, ask questions about the study and I have received satisfactory answers to all my questions.	YES ☐	NO ☐	
I have received enough information about this study.	YES ☐	NO ☐	
I understand that I am free to withdraw from the study up to a certain point and that if I do withdraw this will not affect my future medical care.	YES ☐	NO ☐	
I provide consent for the study team to access my medical records as part of this study.	YES ☐	NO ☐	
I agree to allow the researchers access to my archived tumour tissue to allow for the identification human Papillomavirus (HPV) types.	YES ☐	NO ☐	
I agree to allow the researchers access to my archived tumour tissue for the detection of tumour (somatic) mutations within the tumour DNA.	YES ☐	NO ☐	
I consent to take part in this research study having been fully informed of the risks, benefits and purpose of the study.	YES ☐	NO ☐	

I give my explicit consent to have my data processed as part of this research study.	YES □	NO □	

Participant's Name (Block Capitals):	
Participant's Signature:	
Date:	

To be completed by the RESEARCHER:

I have fully explained the purpose and nature (including benefits and risks) of this study to the participant in a way that he/she could understand. I have invited him/her to ask questions on any aspect of the study.	*YES □*	*NO □*
I confirm that I have given a copy of the information leaflet and consent form to the participant.	YES □	NO □

Researcher's Name (Block Capitals):	
Researcher's Title & Qualifications:	
Researcher's Signature:	
Date:	

Appendix 2: HPV Genotyping

Appendix 2.1: Thermo Fisher Scientific Ion AmpliSeq Designer (Ion Torrent™ by Thermo Fisher Scientific) - Custom Specified Panel - IAD180516

Name		Chr_Start	Chr_End	Amplicons	Total Bases	Covered Bases	Missed Bases	Overall Coverage	Exons
BTF3	BTF3_chr5_72794250_72801448_NM_001207	6909	6910	1	1	1	0	1	n/a
HPV11_E7	HPV11LR_M14119	680	690	1	10	10	0	1	n/a
HPV11_L1	HPV11LR_M14119	6717	6758	1	41	41	0	1	n/a
HPV16_E6_1	HPV16HR_K02718	200	210	1	10	10	0	1	n/a
HPV16_E6_2	HPV16HR_K02718	400	410	1	10	10	0	1	n/a
HPV16_E7	HPV16HR_K02718	710	720	1	10	10	0	1	n/a
HPV16_E2_1	HPV16HR_K02718	2915	2925	1	10	10	0	1	n/a
HPV16_E2_2	HPV16HR_K02718	3200	3210	1	10	10	0	1	n/a
HPV16_E2_3	HPV16HR_K02718	3735	3736	1	1	1	0	1	n/a
HPV16_L1	HPV16HR_K02718	6592	6633	1	41	41	0	1	n/a
HPV18_E7	HPV18HR_X05015	750	760	1	10	10	0	1	n/a
HPV18_L1	HPV18HR_X05015	6568	6609	1	41	41	0	1	n/a
HPV26_E7	HPV26pHR_X74472	713	723	1	10	10	0	1	n/a
HPV26_L1	HPV26pHR_X74472	6543	6584	1	41	41	0	1	n/a
HPV30_E7	HPV30pHR_X74474	724	734	1	10	10	0	1	n/a
HPV30_L1	HPV30pHR_X74474	6587	6628	1	41	41	0	1	n/a
HPV31_E7	HPV31HR_J04353	708	718	1	10	10	0	1	n/a
HPV31_L1	HPV31HR_J04353	6510	6551	1	41	41	0	1	n/a

HPV33_E7	HPV33HR_M12732	719	727	1	8	8	0	1	n/a
HPV33_L1	HPV33HR_M12732	6549	6590	1	41	41	0	1	n/a
HPV34_E7	HPV34pHR_X74476	697	707	1	10	10	0	1	n/a
HPV34_L1	HPV34pHR_X74476	6454	6495	1	41	41	0	1	n/a
HPV35_E7	HPV35HR_X74477	712	722	1	10	10	0	1	n/a
HPV35_L1	HPV35HR_X74477	6550	6591	1	41	41	0	1	n/a
HPV39_E7	HPV39HR_M62849	755	765	1	10	10	0	1	n/a
HPV39_L1	HPV39HR_M62849	6595	6636	1	41	41	0	1	n/a
HPV45_E7	HPV45HR_X74479	750	760	1	10	10	0	1	n/a
HPV45_L1	HPV45HR_X74479	6577	6608	1	31	31	0	1	n/a
HPV51_E7	HPV51HR_M62877	710	720	1	10	10	0	1	n/a
HPV51_L1	HPV51HR_M62877	6481	6512	1	31	31	0	1	n/a
HPV52_E7	HPV52HR_X74481	703	713	1	10	10	0	1	n/a
HPV52_L1	HPV52HR_X74481	6613	6654	1	41	41	0	1	n/a
HPV53_E7	HPV53pHR_X74482	727	737	1	10	10	0	1	n/a
HPV53_L1	HPV53pHR_X74482	6604	6645	1	41	41	0	1	n/a
HPV56_E7	HPV56HR_X74483	730	740	1	10	10	0	1	n/a
HPV56_L1	HPV56HR_X74483	6549	6590	1	41	41	0	1	n/a
HPV58_E7	HPV58HR_D90400	722	732	1	10	10	0	1	n/a

HPV58_L1	HPV58HR_D90400	6598	6639	1	41	41	0	1	n/a
HPV59_E7	HPV59HR_X77858	703	713	1	10	10	0	1	n/a
HPV59_L1	HPV59HR_X77858	6561	6602	1	41	41	0	1	n/a
HPV66_E7	HPV66pHR_U31794	730	740	1	10	10	0	1	n/a
HPV66_L1	HPV66pHR_U31794	6599	6640	1	41	41	0	1	n/a
HPV67_E7	HPV67pHR_D21208	712	722	1	10	10	0	1	n/a
HPV67_L1	HPV67pHR_D21208	6574	6615	1	41	41	0	1	n/a
HPV68_E7	HPV68HR_DQ080079	650	660	1	10	10	0	1	n/a
HPV68_L1	HPV68HR_DQ080079	6443	6484	1	41	41	0	1	n/a
HPV69_E7	HPV69pHR_AB027020	721	731	1	10	10	0	1	n/a
HPV69_L1	HPV69pHR_AB027020	6499	6540	1	41	41	0	1	n/a
HPV6_E7	HPV6LR_X00203	678	688	1	10	10	0	1	n/a
HPV6_L1	HPV6LR_X00203	6732	6773	1	41	41	0	1	n/a
HPV70_E7	HPV70pHR_U21941	756	767	1	11	11	0	1	n/a
HPV70_L1	HPV70pHR_U21941	6539	6580	1	41	41	0	1	n/a
HPV73_E7	HPV73pHR_X94165	696	706	1	10	10	0	1	n/a
HPV73_L1	HPV73pHR_X94165	6423	6464	1	41	41	0	1	n/a
HPV82_E7	HPV82pHR_AB027021	716	726	1	10	10	0	1	n/a
HPV82_L1	HPV82pHR_AB027021	6531	6562	1	31	31	0	1	n/a

HPV85_E7	HPV85pHR_AF131950	750	760	1	10	10	0	1	n/a
HPV85_L1	HPV85pHR_AF131950	6565	6606	1	41	41	0	1	n/a
HPV97_E7	HPV97pHR_DQ080080	646	656	1	10	10	0	1	n/a
HPV97_L1	HPV97pHR_DQ080080	6449	6490	1	41	41	0	1	n/a
RPL32	RPL32_chr3_12876444_12883081_NM_000994	5491	5492	1	1	1	0	1	n/a
RPS27A	RPS27A_chr2_55459039_55462989_NM001135592	3663	3664	1	1	1	0	1	n/a
UBA52	UBA52_chr19_18682614_18688270_NM_003333	3398	3399	1	1	1	0	1	n/a

Appendix 3: Cancer Staging

Appendix 3.1: AJCC/UICC 7th Edition TNM and Staging

TNM Staging Classification of Oropharyngeal Cancers	
7th Edition	
T	
1	Tumour 2cm or less in greatest dimension
2	Tumour more than 2cm but not more than 4cm in greatest dimension
3	Tumour more than 4cm in greatest dimension
4a	Tumour invades the larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible
4b	Tumour invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases carotid artery.
N	
X	Regional lymph nodes cannot be assessed
0	No regional lymph node metastasis
1	Metastasis in a single ipsilateral node, 3cm or less in greatest dimension
2	Metastasis in a single ipsilateral node, more than 3cm but not more than 6cm in greatest dimension, or in multiple ipsilateral lymph nodes, none more than 6cm in greatest, or in bilateral or contralateral lymph nodes, none more than 6cm in greatest dimension
2a	Metastasis in a single ipsilateral lymph node, more than 3cm but not more than 6cm in greatest dimension
2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6cm in greatest dimension
2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6cm in greatest dimension
3	Metastasis in a lymph node more than 6cm in greatest dimension
M	
X	Distant metastasis cannot be assessed
0	No distant metastasis
1	Distant metastasis

AJCC/UICC 7th Edition Staging Classification of Oropharyngeal Cancers			
Stage 0	Tis	**N0**	M0
Stage I	T1	**N0**	M0
Stage II	T2	**N0**	M0
Stage III	T3	**N0**	M0
	T1	**N1**	M0
	T2	**N1**	M0
	T3	**N1**	M0
Stage IVA	T4a	**N0**	M0
	T4a	**N1**	M0
	T1	**N2**	M0
	T2	**N2**	M0
	T3	**N2**	M0
	T4a	**N2**	M0
Stage IVB	T4b	**Any N**	M0
	Any T	**N3**	M0
Stage IVC	Any T	**Any N**	M1

Appendix 4: HPV Genotyping Results

Appendix 4.1: HPV Genotyping Results from Ion Torrent Next Generation Sequencing Read Counts

Reference Name	10B	11B	14B	15R	16B	17R	19B	1B	20B
BTF3_chr5	1943	10477	601	7275	11654	7725	2469	4431	3743
RPL32_chr3	1121	10823	548	6794	6340	3921	2828	1884	2231
RPS27A_chr2	1010	7100	185	2216	3659	2245	1067	1526	1413
UBA52_chr19	726	6763	322	3238	2367	1897	1583	945	1062
HPV16HR_K02718	477298	224	25368	24935	37387	1913	35	12606	541
HPV35HR_X74477	10	0	0	2	0	2	0	2	0
HPV59HR_X77858	3	3	1	8	1	2	1	0	2
HPV11LR_M14119	0	0	0	0	0	0	0	0	0
HPV18HR_X05015	0	0	0	0	0	0	0	0	0
HPV26pHR_X74472	0	0	0	0	0	0	0	0	0
HPV30pHR_X74474	0	0	0	0	0	0	0	0	0
HPV31HR_J04353	0	0	0	0	0	0	0	0	0
HPV33HR_M12732	0	357099	0	0	0	6	2271	0	0
HPV34pHR_X74476	0	0	0	0	0	0	0	0	0
HPV39HR_M62849	0	0	0	20109	0	0	0	0	0
HPV45HR_X74479	0	0	1	0	0	0	0	0	0
HPV51HR_M62877	0	0	0	0	0	0	0	0	0
HPV52HR_X74481	0	0	0	0	0	0	0	0	2
HPV53pHR_X74482	0	0	0	0	0	0	0	0	0
HPV56HR_X74483	0	0	0	0	0	0	0	0	0
HPV58HR_D90400	0	0	0	0	0	0	0	0	0
HPV66pHR_U31794	0	0	0	0	0	0	0	0	0
HPV67pHR_D21208	0	0	0	0	0	0	0	0	0
HPV68HR_DQ080079	0	0	0	0	0	0	0	0	0

HPV69pHR_AB027020	0	0	0	0	0	0	0	1	0
HPV6LR_X00203	0	0	0	0	0	0	0	0	0
HPV70pHR_U21941	0	0	0	0	0	0	0	0	0
HPV73pHR_X94165	0	0	0	0	0	0	0	0	0
HPV83_AF151983	0	0	0	0	0	0	0	0	0
HPV85pHR_AF131950	0	0	0	0	0	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0	0	0	0	0	0

Reference Name	22R	27B	28B	29B	2B	30B	31B	32B	33B
BTF3_chr5	386	18549	4552	2558	1550	52566	3109	11931	1612
RPL32_chr3	208	18737	3551	678	483	24436	1373	7413	636
RPS27A_chr2	117	10764	1209	990	880	28106	1157	6926	747
UBA52_chr19	116	12535	1466	396	286	10377	727	5367	412
HPV16HR_K02718	295	41	705	3233	117	6415	196926	270492	200
HPV35HR_X74477	0	0	0	0	0	4	1	0	0
HPV59HR_X77858	2	2	0	3	4	4	0	1	3
HPV11LR_M14119	0	0	0	0	0	0	0	0	0
HPV18HR_X05015	0	0	0	0	0	0	0	0	0
HPV26pHR_X74472	0	0	0	0	0	0	0	0	0
HPV30pHR_X74474	1	0	0	1	0	0	0	0	0
HPV31HR_J04353	0	0	0	0	0	0	0	0	0
HPV33HR_M12732	1	0	0	0	0	2	0	0	2
HPV34pHR_X74476	0	0	0	0	0	0	0	0	0
HPV39HR_M62849	0	0	0	0	0	0	0	0	0
HPV45HR_X74479	0	0	0	0	0	0	0	0	0
HPV51HR_M62877	0	0	0	0	0	0	0	0	0
HPV52HR_X74481	0	0	0	0	0	0	0	0	1
HPV53pHR_X74482	0	0	0	0	0	0	0	0	0
HPV56HR_X74483	0	0	0	0	0	0	0	1	0
HPV58HR_D90400	0	0	0	0	0	0	0	0	0
HPV66pHR_U31794	0	0	0	0	0	0	0	0	0
HPV67pHR_D21208	0	0	0	0	0	0	0	0	0
HPV68HR_DQ080079	0	0	0	0	0	0	0	0	0

HPV69pHR_AB027020	0	0	0	0	0	0	0	0	0
HPV6LR_X00203	0	0	0	0	0	0	0	0	0
HPV70pHR_U21941	0	0	0	0	0	1	0	0	0
HPV73pHR_X94165	0	0	0	0	0	0	0	0	0
HPV83_AF151983	0	0	0	0	0	0	0	0	0
HPV85pHR_AF131950	0	0	0	0	0	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0	0	0	0	0	0

Reference Name	36B	37B	38B	3R	40B	41R	42B	43B	44B
BTF3_chr5	290	4534	721	7504	5	14182	52	6929	7020
RPL32_chr3	403	1856	427	2969	0	3541	23	2586	4427
RPS27A_chr2	118	955	272	1895	0	3669	19	2100	2941
UBA52_chr19	152	533	163	1241	3	1334	17	1283	2707
HPV16HR_K02718	11209	323613	137	70	17	37573	32563	133064	534
HPV35HR_X74477	5	0	0	1	0	0	0	4	0
HPV59HR_X77858	0	2	0	0	1	2	0	3	1
HPV11LR_M14119	0	0	0	0	0	0	0	0	0
HPV18HR_X05015	0	0	0	0	0	0	0	0	0
HPV26pHR_X74472	0	0	0	0	0	0	0	0	0
HPV30pHR_X74474	0	0	0	0	0	0	0	0	0
HPV31HR_J04353	0	0	0	0	0	0	0	0	0
HPV33HR_M12732	0	0	0	9	0	0	0	0	0
HPV34pHR_X74476	0	0	0	0	0	0	0	0	0
HPV39HR_M62849	1	0	0	0	0	1	0	0	0
HPV45HR_X74479	0	0	0	0	0	0	0	0	0
HPV51HR_M62877	0	0	0	0	0	0	0	0	0
HPV52HR_X74481	0	0	0	0	0	0	0	0	0
HPV53pHR_X74482	0	0	0	0	0	0	0	0	0
HPV56HR_X74483	0	0	0	0	0	0	0	0	0
HPV58HR_D90400	0	0	0	0	0	0	0	0	0
HPV66pHR_U31794	0	0	0	0	0	0	0	0	0
HPV67pHR_D21208	0	0	0	0	0	0	0	0	0
HPV68HR_DQ080079	0	0	0	0	0	0	0	0	0

HPV69pHR_AB027020	0	0	0	0	0	0	0	0	0
HPV6LR_X00203	0	0	0	0	0	0	0	0	0
HPV70pHR_U21941	0	0	0	0	0	0	0	0	0
HPV73pHR_X94165	0	0	0	0	0	0	0	0	0
HPV83_AF151983	0	0	0	0	0	0	0	0	0
HPV85pHR_AF131950	0	0	0	0	0	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0	0	0	0	0	0

Reference Name	45B	46B	48B	49B	4R	50B	51B	53B	54B
BTF3_chr5	8697	436	4770	5303	18625	14875	1333	17516	2277
RPL32_chr3	2772	775	1772	2419	13168	18021	464	4827	838
RPS27A_chr2	3091	100	926	1795	6391	8758	436	5557	547
UBA52_chr19	1154	268	570	1070	6298	10188	199	1492	261
HPV16HR_K02718	17024	19774	27209	191126	120	776	13340	337184	64342
HPV35HR_X74477	1	0	0	0	0	1	21	0	0
HPV59HR_X77858	2	0	3	5	1	2	6	2	0
HPV11LR_M14119	0	0	0	0	0	0	0	0	0
HPV18HR_X05015	0	0	0	0	0	0	0	0	0
HPV26pHR_X74472	0	0	0	0	0	0	0	0	0
HPV30pHR_X74474	0	0	0	0	0	0	0	0	0
HPV31HR_J04353	0	0	0	0	0	0	0	0	0
HPV33HR_M12732	0	1	1	202	0	49	0	0	1
HPV34pHR_X74476	0	0	0	0	0	0	0	0	0
HPV39HR_M62849	0	0	0	0	0	0	0	0	0
HPV45HR_X74479	0	0	0	1	0	0	0	0	0
HPV51HR_M62877	0	0	0	0	0	0	0	0	0
HPV52HR_X74481	0	0	0	0	0	0	0	0	0
HPV53pHR_X74482	0	0	0	0	0	0	0	0	0
HPV56HR_X74483	0	0	0	64	0	0	0	2	0
HPV58HR_D90400	0	0	0	0	0	0	0	0	0
HPV66pHR_U31794	0	0	0	0	0	0	0	0	0
HPV67pHR_D21208	0	0	0	0	0	0	0	0	0
HPV68HR_DQ080079	0	0	0	0	0	0	0	0	0

HPV69pHR_AB027020	0	0	0	0	0	0	0	0	0
HPV6LR_X00203	0	0	0	0	0	0	0	0	0
HPV70pHR_U21941	1	0	0	0	0	0	0	0	0
HPV73pHR_X94165	0	0	0	0	0	0	0	0	0
HPV83_AF151983	0	0	0	0	0	0	0	0	0
HPV85pHR_AF131950	0	0	0	0	0	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0	0	0	0	0	0

Reference Name	55B	56B	57B	58B	59B	5R	60B	61B	62B
BTF3_chr5	3254	237	1855	12063	2226	4238	238	7878	7070
RPL32_chr3	2124	61	2935	2628	1123	1967	505	2856	2483
RPS27A_chr2	1225	51	754	4454	1074	1035	102	2914	2430
UBA52_chr19	793	12	1023	1453	445	871	307	1215	1432
HPV16HR_K02718	17496	2189	368	164	270353	354055	157460	242	76670
HPV35HR_X74477	0	0	0	21	0	0	0	8853	3
HPV59HR_X77858	3	0	0	2	1	3	0	5	6
HPV11LR_M14119	0	0	0	1	0	0	0	0	0
HPV18HR_X05015	0	0	0	0	0	0	0	0	0
HPV26pHR_X74472	0	0	0	0	0	0	0	0	0
HPV30pHR_X74474	0	0	0	0	0	0	0	0	0
HPV31HR_J04353	0	0	0	0	0	0	0	0	0
HPV33HR_M12732	18	0	0	2442	0	0	0	0	0
HPV34pHR_X74476	0	0	0	0	0	0	0	0	0
HPV39HR_M62849	0	0	0	6	0	0	0	0	14
HPV45HR_X74479	0	0	0	0	0	0	0	0	0
HPV51HR_M62877	0	0	0	0	0	0	0	0	0
HPV52HR_X74481	0	0	0	0	1	0	0	0	0
HPV53pHR_X74482	0	0	0	0	0	0	0	0	0
HPV56HR_X74483	0	0	0	0	0	0	0	0	0
HPV58HR_D90400	0	0	0	0	0	0	0	0	0
HPV66pHR_U31794	0	0	0	0	0	0	0	0	0
HPV67pHR_D21208	0	0	0	0	0	0	0	0	0
HPV68HR_DQ080079	0	0	0	0	0	0	0	0	0

HPV69pHR_AB027020	0	0	0	0	0	0	0	0	0
HPV6LR_X00203	0	0	0	0	0	0	0	0	0
HPV70pHR_U21941	0	0	0	0	0	0	0	0	0
HPV73pHR_X94165	0	0	0	0	0	0	0	0	0
HPV83_AF151983	0	0	0	0	0	0	0	0	0
HPV85pHR_AF131950	0	0	0	0	0	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0	0	0	0	0	0

Reference Name	63B	65B	66R	67R	69B	6B	70B	71B	72B
BTF3_chr5	15824	7869	5326	18138	2871	1360	13115	3802	5432
RPL32_chr3	7903	2875	2420	7568	1698	416	11508	3050	4003
RPS27A_chr2	6439	2665	2735	7883	1039	754	5898	1642	2274
UBA52_chr19	4235	1237	982	2811	809	149	4210	1263	2349
HPV16HR_K02718	633	106249	20	97459	19316	152	67719	19320	12144
HPV35HR_X74477	3	6	0	0	0	0	0	0	0
HPV59HR_X77858	1	0	1	1	1	1	1	0	1
HPV11LR_M14119	0	0	0	0	0	0	0	0	0
HPV18HR_X05015	0	0	0	0	0	0	0	0	0
HPV26pHR_X74472	0	0	0	0	0	0	0	0	0
HPV30pHR_X74474	0	0	0	0	0	0	0	0	0
HPV31HR_J04353	0	0	0	0	0	0	0	0	0
HPV33HR_M12732	0	0	0	0	0	2	0	0	0
HPV34pHR_X74476	0	0	0	0	0	0	0	0	0
HPV39HR_M62849	0	0	0	0	0	0	0	0	0
HPV45HR_X74479	0	0	0	0	0	0	0	0	0
HPV51HR_M62877	0	0	0	0	0	0	0	0	0
HPV52HR_X74481	0	0	0	0	0	0	0	0	0
HPV53pHR_X74482	0	0	1	0	0	0	0	0	0
HPV56HR_X74483	0	0	0	0	0	0	0	0	0
HPV58HR_D90400	0	0	0	0	0	0	0	0	0
HPV66pHR_U31794	0	0	0	0	0	0	0	0	0
HPV67pHR_D21208	0	0	0	0	0	0	0	0	0
HPV68HR_DQ080079	0	0	0	0	0	0	0	0	0

HPV69pHR_AB027020	0	2	0	0	0	0	0	0	0
HPV6LR_X00203	0	0	0	0	0	0	0	0	0
HPV70pHR_U21941	0	0	0	0	0	0	0	0	0
HPV73pHR_X94165	0	0	0	0	0	0	0	0	0
HPV83_AF151983	0	0	0	0	0	0	0	0	0
HPV85pHR_AF131950	0	0	0	0	0	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0	0	0	0	0	0

Reference Name	73B	74B	78R	79B	7B	80B	81B	82B	84B
BTF3_chr5	8990	23414	6374	12311	6538	3205	15404	5774	1782
RPL32_chr3	4391	6775	3294	4427	2355	1240	6988	6222	1776
RPS27A_chr2	3465	8499	2401	4852	2132	1140	5805	2260	691
UBA52_chr19	1934	2508	1237	2113	1172	491	1511	2452	767
HPV16HR_K02718	122135	394422	110	2171	205951	220306	233640	323148	16388
HPV35HR_X74477	0	0	0	0	0	0	0	0	0
HPV59HR_X77858	1	5	14	3	0	1	0	2	1
HPV11LR_M14119	0	0	0	0	0	0	0	0	0
HPV18HR_X05015	0	0	0	1	0	0	0	0	0
HPV26pHR_X74472	0	0	0	0	0	0	0	0	0
HPV30pHR_X74474	0	0	0	0	0	0	0	0	0
HPV31HR_J04353	0	0	0	0	0	0	0	0	0
HPV33HR_M12732	0	0	5	0	0	0	30	0	0
HPV34pHR_X74476	0	0	0	0	0	0	0	0	0
HPV39HR_M62849	0	0	0	0	0	0	0	0	0
HPV45HR_X74479	0	0	0	0	0	0	0	0	0
HPV51HR_M62877	0	0	0	0	0	0	0	0	0
HPV52HR_X74481	0	0	0	0	0	0	0	0	0
HPV53pHR_X74482	0	0	0	0	0	0	0	0	0
HPV56HR_X74483	0	0	0	0	0	0	1	1	0
HPV58HR_D90400	0	0	0	0	0	0	0	0	0
HPV66pHR_U31794	0	0	0	0	0	0	0	0	0
HPV67pHR_D21208	0	0	0	0	0	0	0	0	0
HPV68HR_DQ080079	0	0	0	0	0	0	0	0	0

HPV69pHR_AB027020	0	1	0	0	0	0	0	0	0
HPV6LR_X00203	0	0	0	0	0	0	0	0	0
HPV70pHR_U21941	0	0	0	0	0	0	0	0	0
HPV73pHR_X94165	0	0	0	0	0	0	0	0	0
HPV83_AF151983	0	0	0	0	0	0	0	0	0
HPV85pHR_AF131950	0	3	0	0	0	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0	0	0	0	0	0

Reference Name	85B	86B	8R	9B
BTF3_chr5	26567	6564	24520	1899
RPL32_chr3	8811	3590	9970	1112
RPS27A_chr2	8597	3585	7045	645
UBA52_chr19	3000	1418	3471	570
HPV16HR_K02718	39923	79977	262705	111084
HPV35HR_X74477	0	0	0	1
HPV59HR_X77858	1	4	1	0
HPV11LR_M14119	0	0	0	0
HPV18HR_X05015	0	0	0	0
HPV26pHR_X74472	0	0	0	0
HPV30pHR_X74474	0	0	0	0
HPV31HR_J04353	0	0	0	0
HPV33HR_M12732	5	0	42	0
HPV34pHR_X74476	0	0	0	0
HPV39HR_M62849	0	0	0	0
HPV45HR_X74479	0	0	0	0
HPV51HR_M62877	0	0	0	0
HPV52HR_X74481	0	0	0	0
HPV53pHR_X74482	0	0	0	0
HPV56HR_X74483	0	0	0	0
HPV58HR_D90400	0	0	0	0
HPV66pHR_U31794	0	0	0	0
HPV67pHR_D21208	0	0	0	0
HPV68HR_DQ080079	0	0	0	0
HPV69pHR_AB027020	1	0	0	0

HPV6LR_X00203	0	0	0	0
HPV70pHR_U21941	0	0	0	0
HPV73pHR_X94165	0	0	0	0
HPV83_AF151983	0	0	0	0
HPV85pHR_AF131950	0	0	0	0
HPV97pHR_DQ080080	0	0	0	0