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Human papillomavirus (HPV) associated oropharyngeal cancer: Case prevalence, diagnosis, and the potential for screening in New Zealand.

A thesis presented in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy in Public Health

at Massey University,

Manawatu, New Zealand.

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2018

Abstract

The incidence of oropharyngeal cancer (OPC) is increasing in developed countries, with many cases caused by Human Papilloma Virus (HPV). Since the mid-1990s, the incidence of OPC in New Zealand has almost quadrupled, but the role of HPV is uncertain. Thus, the objectives of this research were to establish the burden of HPV positive OPC in New Zealand, investigate diagnostic methods, early detection through PCR and cytology, and risk factors in a pre-vaccine population.

The results presented in this thesis demonstrate an increased prevalence of HPV positive OPC from 61.9% in 1996-98 to 87.5% in 2010-12 in the study population. HPV 16 was responsible for 98.5% of HPV positive OPC and results from the multivariable model showed an HPV positive patient was more likely to be aged under 60 years old and diagnosed in 2010-12. Descriptive Analysis of questionnaire data from OPC patients found that having ever given oral sex was the most significant risk factor for having an HPV (p16) positive tumour.

Comparison of p16, CK19, and HPV 16 DNA, RNA detection and viral load revealed ten cases in which the HPV status was incorrectly classified based on p16 alone, showing the need for clearer guidelines around the reporting of p16 results. These results are of particular importance as de-escalated therapies for HPV positive cases are under investigation. Moreover, viral nucleic acid and cytological abnormalities were detectable in brushings taken from conscious OPC patients. These previously undescribed cellular changes are comparable to cervical precancerous lesions and

showed a continuum of dysplasia in p16 positive cases only. This warrants further investigation.

Overall, this research has shown HPV positive OPC is a significant burden on the New Zealand health system and its incidence is increasing, thus supporting the recent inclusion of males into the nationally funded immunization schedule for Gardasil®9. However, OPC cases will continue to increase until the current vaccinated cohort reaches middle age. It is crucial that until this time, we focus on the early detection, improved diagnostics and reduced morbidities from treatment in the current pre-vaccine population.

Acknowledgements

First and foremost I would like to thank my supervisors, you have all believed in me throughout this project and I have learnt so much from all of you. To Laryssa Howe, thank you for always having an open door and time for a quick question or a chat. Not to mention your glass half full approach after any problems in the lab. Jackie Benschop thank you for all your guidance and your ability to explain all things statistics in a way I could understand. To Bruce Lockett, thank you for your attention to detail and unwavering enthusiasm when presented with another batch of very average looking E7 immunohistochemistry slides.

I am grateful for the financial support from the Massey University Doctoral scholarship I have received during the PhD. Thank you also to the McGeorge foundation, the Palmerston North Medical Research Foundation, the Hawkes Bay Medical Research Foundation, the Massey University Research fund and the IVABS postgraduate funds. This research would not have been possible without the funds you awarded us.

To all the participants involved in this research thank you for taking the time to be involved and in particular those who piloted and completed the questionnaire for us. Your openness and willingness to share your experience has made such a difference and we appreciate all that you shared with us.

A big thank you to everyone involved in collecting the brushings, in particular Wendy Mann and Dr Dean Ruske for the steady stream of samples that came in. Toni Webber, for all the fortnightly emails so I could catch all the local patients. Finally to Amy, Erin and Kim for their help in taking the brushings, we got it down to a fine art!

Thank you to Drs Aneesh Kumar and Nikolay Nedev for teaching me tricks for how to visualise and sample the tonsils while keeping the tongue out of the way. Those tips made a huge difference for both myself and the patients being sampled.

Thank you to Dr Julian White for convincing so many other ENT surgeons to come on board with the project, and for your input into the questionnaire and other clinical aspects of the project.

To all the staff at MedLab Central who have helped with the project. TC, Ursula, and Savannah for your help screening/reading all the slides. Viv for supplying me with all my Cytobrushes, and Herman for allowing me to use all the lab equipment for my tonsillar brushings, and letting me raid the supplies of Thin Prep vials and PreservCyt. Lastly to Jackie and Ruth thank you for all your work preparing slides and cutting slices to be used in PCR work. The job was much bigger than first anticipated and I think I'm overdue in providing another cake. Also an extra thank you to Ruth for your efforts with the E7 assay, it was trying to say the least.

So many people contributed to this project along the way and I'm very grateful for all of the contributions, thank you very much. Dr Michael Dictor for providing control HPV samples from Sweden with no hesitation. Gerard Prinsen for the introduction into qualitative research and help with the free text comments. Jeroen Douwes for the assistance with our occupational exposures data, and the sharing of the New Zealand working survey exposures. Ahmed Fayaz, for the creation of a great looking and very easy to use database for the questionnaire responses. Dr Claire Hardie, for her peer review and input into the study design. Maureen Holdaway for the Maori consultation for the project. Liz Burrows for getting reagents ready for me and making sure there

were plenty of lab supplies for the long weekends of DNA extractions. Neil Blackwell from Hologic for the donation of the filters for our brushings. All the staff at labs throughout the country that retrieved blocks for the first study for me. Finally, thank you to all the staff at DHB research offices and HDEC who have been so helpful throughout the whole project.

To the 2.12 office girls, past and present. Thank you for the banter and support. Not to mention the puzzle time, and vending machine runs. It's been great sharing the PhD journey with you all.

To my friends Karina and Laura, thank you for all your late notice babysitting so I could recruit participants. You both made so much possible. Thank you also for the impromptu dinners and wines that followed, they sure helped keep some balance during the crazy times.

Thank you to my parents for always showing an interest and giving me your support. In particular my mum Jackie, your babysitting Max on Tuesdays for the last six months has made the world of difference to getting this thing done. Not to mention the meals you prepared for us and cleaning and gardening you did too. You really have been a life saver, and we appreciate it more than you know.

To my boys Scott and Max you have been the best support crew. My little man, Max thank you for listening to all my presentations and being such an angel all the times I took you to meetings or to pick up samples. To my husband Scott, thank you for being my rock, the last few years have thrown so much at us and we've made it, the best really is yet to come. Thank you also for learning some lab words and phrases so you could ask about my day in detail, you always know how to make me smile.

List of publications and presentations

Publications

Lucas-Roxburgh, R., Benschop, J., Lockett, B., van den Heever, U., Williams, R., & Howe, L. (2017). The prevalence of human papillomavirus in oropharyngeal cancer in a New Zealand population. *PLoS ONE, 12*(10), 1-13. doi: 10.1371/journal.pone.0186424

Conference Presentations

Lucas-Roxburgh, R., Benschop, J., Lockett, B. & Howe, L. HPV 16 mRNA and DNA detection, viral load, and cytology in tonsillar brushings from conscious oropharyngeal cancer patients (poster presentation). HPV 2018, 32nd International Papillomavirus Conference. October 2-6, 2018, Sydney, Australia.

Lucas-Roxburgh, R., Benschop, J., Lockett, B. & Howe, L. Descriptive analysis of a pre-vaccine population of oropharyngeal cancer patients in New Zealand (poster presentation). HPV 2018, 32nd International Papillomavirus Conference. October 2-6, 2018, Sydney, Australia.

Lucas-Roxburgh R., Benschop J., Lockett B., van den Heever U., Williams R. & Howe, L. Prevalence of HPV positive oropharyngeal cancer in New Zealand. 10th New Zealand Immunization conference and pre-conference workshop. September 8-9, 2017 Wellington, New Zealand.

Lucas-Roxburgh, R., Benschop, J., Lockett, B., Williams, R. & Howe, L. Detection of human papillomavirus (HPV) 16 E7 protein in oropharyngeal cancer biopsies with

focally positive p16 staining (poster presentation). HPV 2017, 31st International Papillomavirus Conference. February 28 – March 4, 2017, Cape Town, South Africa.

Lucas-Roxburgh, R., Benschop, J., Lockett, B., Williams, R. & Howe, L. Prevalence of HPV positive oropharyngeal cancer in New Zealand (poster presentation). HPV 2017, 31st International Papillomavirus Conference. February 28 – March 4, 2017, Cape Town, South Africa.

Lucas-Roxburgh, R. How old is too old: p16 testing on Oropharyngeal biopsies. Roche Histopathology User Group. March 22-28, 2015, Taupo, New Zealand.

Lucas-Roxburgh, R. (2015) Connect four: Using HRM to detect HPV genotypes in Oropharyngeal Cancer. Roche Molecular Diagnostics User Group. March 22-28, 2015, Taupo, New Zealand.

Invited speaker presentations

Lucas-Roxburgh, R. HPV and associated cancers / The burden of oropharyngeal cancer in New Zealand. Whanganui Inter-Professional Education (WIPE): HPV and oropharyngeal cancers. April 3, 2018, Whanganui, New Zealand.

Lucas-Roxburgh, R. HPV in oropharyngeal cancer. The immunisation advisory centre professional development day. May 9, 2018, Wellington, New Zealand.

Lucas-Roxburgh R. Prevalence of HPV positive oropharyngeal cancer in New Zealand. National Cervical Screening Program Clinical Update. November 29, 2017, Palmerston North, New Zealand.

Lucas-Roxburgh, R. HPV-associated Oropharyngeal Cancer in New Zealand. Royal College of Pathologists of Australasia (RCPA) Annual Scientific Meeting. September 23-25, 2016, Napier, New Zealand.

Lucas-Roxburgh, R. HPV Associated Oropharyngeal Squamous Cell Carcinoma. New Zealand Institute of Medical Laboratory Science (NZIMLS) South Pacific Congress. August 17-21, 2015, Auckland, New Zealand.

Awards

2017 Massey University Three minute thesis (3MT) – People's choice award.

2015 Roche User Group, Histopathology – Best Scientific Content: How old is too old: p16 testing on Oropharyngeal biopsies.

Preface

An article (of the non-academic, non-peer reviewed, and glossy magazine variety) sparked my interest in HPV related head and neck cancers. The article reported on the increase in the number of females with HPV related head and neck cancers in the United States and warned of the dangers of oral sex. Armed with only a cytologist's knowledge of cervical HPV, questions were automatically generated. Is there some sort of transformation zone? And could we screen for this disease? During the PhD, these and many other questions were raised and answered, and a whole new set of questions were born.

Thesis structure and format

This thesis aims to assess the burden of HPV positive oropharyngeal cancer in New Zealand, and investigate diagnostic methods and possibilities for early detection in a pre-vaccine population. This will be undertaken by addressing each of the following specific aims:

1. To determine the prevalence and genotypes associated with HPV positive oropharyngeal cancer in New Zealand (Chapter 2).
2. To compare HPV detection using a range of biomarkers in oropharyngeal cancer biopsies with variable p16 results (Chapter 3).
3. To describe the demographics and exposures of a pre-vaccine population of oropharyngeal cancer patients (Chapter 4).
4. To use brushings from conscious oropharyngeal cancer patients to investigate the detection of HPV 16, and cytological abnormalities (Chapter 5).

This thesis is written as a series of six interrelated Chapters, one of which (Chapter 2) has been published in a peer reviewed journal (Lucas-Roxburgh *et al*, 2017). Each Chapter is presented in the format of a manuscript for a peer reviewed journal and as a consequence there is some repetition between Chapters. A literature review (Chapter 1) introduces the thesis topic by discussing human papillomavirus and its role in human disease with a focus on oropharyngeal cancer. The thesis concludes with a

general discussion (Chapter 6) which summarizes the results of the research and puts these into context. The relevance of the findings and future directions for research are also described.

All references are listed at the end of the thesis in order to minimise repetition. All cited literature uses the format of PLoS ONE.

Table of contents

Abstract i
Acknowledgements iii
List of publications and presentations vi
Preface ix
Thesis structure and format x
Table of contents xii
List of Figures xvii
List of Tables xviii
Chapter 1 - Review of the literature 1
1.1 Introduction 2
1.2 Characteristics of human papillomavirus 3
1.2.1 Genome 3
1.2.1.1 Early gene expression 3
1.2.1.2 Late gene expression 4
1.2.1.3 Viral oncogene expression 4
1.2.2 Lifecycle 5
1.3 HPV in benign conditions 6
1.3.1 Cutaneous warts 6
1.3.2 Focal epithelial hyperplasia (Heck's disease) 7
1.3.3 Epidermodysplasia verruciformis 8
1.3.4 Genital warts 8
1.4 HPV associated cancers 8
1.4.1 Cervical cancer 9
1.4.2 Other anogenital cancers 11
1.4.3 Head and neck cancer 12
1.5 Oropharyngeal cancer 12
1.5.1 Risk factors for HPV positive oropharyngeal cancer 14
1.5.2 Development of HPV positive oropharyngeal cancer 17
1.6 Laboratory Diagnosis of HPV 19
1.6.1 Polymerase chain reaction 19
1.6.2 In situ hybridisation 21

1.6.3 p16 immunohistochemistry 22
1.6.4 Other biomarkers 25
1.7 Prevention of HPV associated cancers 25
1.7.1 Primary prevention - Vaccination 26
1.7.1.1 Vaccination for HPV positive oropharyngeal cancer 28
1.7.2 Secondary prevention - Screening 28
1.7.2.1 Screening for HPV positive oropharyngeal cancer 29
1.8 Oropharyngeal cancer in New Zealand 31
1.9 Conclusion 32
Chapter 2 - The prevalence of human papillomavirus in oropharyngeal cancer in a New Zealand population 33
2.1 Abstract 34
2.2 Introduction 35
2.3 Materials and methods 36
2.3.1 Participant recruitment and sample collection 36
2.3.2 p16 IHC 38
2.3.3 DNA extraction 39
2.3.4 Beta-globin qPCR 39
2.3.5 HPV 16 qPCR 42
2.3.6 Confirmation of non-HPV 16 types 43
2.3.7 Data handling and statistical analysis 43
2.4 Results 44
2.4.1 Study population 44
2.4.2 p16 IHC 47
2.4.3 Beta-globin, HPV 16, and GP5+/6+ qPCR 48
2.4.4 Statistical analysis 50
2.5 Discussion 52
Chapter 3 - Evaluation of p16 and CK19 immunohistochemistry, nucleic acid detection and viral load in archived formalin fixed paraffin embedded biopsy samples for the detection of HPV in oropharyngeal cancer 59
3.1 Abstract 60
3.2 Introduction 61
3.3 Materials and methods 64
3.3.1 Participant recruitment and sample collection 64
3.3.2 DNA extraction 65

3.3.4 RNA extraction....65
3.3.5 Beta-globin qPCR....66
3.3.6 HPV 16 DNA qPCR and viral load calculations....66
3.3.7 RT-PCR....67
3.3.8 HPV 16 E7 qPCR....67
3.3.9 p16 immunohistochemistry....68
3.3.10 CK19 immunohistochemistry....68
3.3.11 HPV 16 E7 immunohistochemistry....69
3.3.12 Statistical analysis....70
3.4 Results....70
3.4.1 Focally positive p16 specimens....70
3.4.2 HPV 16 DNA and viral load....71
3.4.3 HPV 16 RNA detection....71
3.4.4 CK19 immunohistochemistry....75
3.4.5 HPV 16 E7 immunohistochemistry....76
3.4.6 Test comparisons....76
3.5 Discussion....80
Chapter 4 - Descriptive analysis of a pre-vaccine population of oropharyngeal cancer patients in New Zealand....85
4.1 Abstract....86
4.2 Introduction....87
4.3 Materials and Methods....90
4.3.1 Study design....90
4.3.2 Sample size calculation....91
4.3.3 Questionnaire....92
4.3.4 Laboratory testing....93
4.3.5 Data categorisation and Statistical analyses....93
4.3.6 Comparisons to the New Zealand Working Survey....94
4.3.7 Free text comments....94
4.3.8 Uni- and multivariable Analysis....94
4.4 Results....95
4.4.1 Exposure to hazardous substances....97
4.4.2 Alcohol, Tobacco and marijuana use....100
4.4.3 Sexual behaviours....103

4.4.4 Medical history 106
4.4.5 Symptoms 109
4.4.6 Uni- and multi-variate analysis 110
4.4.7 Inductive content analysis 113
4.5 Discussion........ 113
Chapter 5 - HPV 16 mRNA and DNA detection, viral load, and cytology in tonsillar brushings from conscious oropharyngeal cancer patients........ 119
5.1 Abstract........ 120
5.2 Introduction 121
5.3 Materials and Methods........ 124
5.3.1 Participant recruitment and sample collection. 124
5.3.2 DNA extraction........ 125
5.3.3 RNA extraction 126
5.3.4 Beta-globin qPCR........ 126
5.3.5 HPV 16 E7 qPCR........ 126
5.3.6 RT-PCR........ 127
5.3.7 Viral load calculations 127
5.3.8 Liquid based cytology preparations........ 128
5.3.9 Statistical analysis 129
5.4 Results........ 129
5.4.1 Sample collection. 129
5.4.2 HPV 16 E7 DNA detection 130
5.4.3 Viral load 130
5.4.4 HPV 16 E7 RNA detection........ 131
5.4.5 Liquid based cytology........ 132
5.4.6 Results by tumour site 133
5.5 Discussion........ 135
Chapter 6 - General Discussion........ 141
6.1 Introduction 142
6.2 Summary of results 142
6.3 Current difficulties in the determination of HPV status in oropharyngeal cancer 143
6.4 The role of vaccination in prevention of oropharyngeal cancer........ 146
6.5 Possibilities for the early detection of HPV positive oropharyngeal cancer........ 150
6.6 Risk factors for oropharyngeal cancer 155

6.7 Conclusions 158
References 159
Appendix 1 – Published PloS ONE article 170
Appendix 2 – HDEC approval letter (14/STH/128) 183
Appendix 3 – PISCF Chapter two study 187
Appendix 4 – Beta-Globin qPCR SOP 193
Appendix 5 - HPV 16 qPCR SOP 195
Appendix 6 – GP5+/6+ qPCR SOP 197
Appendix 7 – HPV 16 E7 qPCR SOP 199
Appendix 8 – HDEC approval letter (15/NTB/155) 201
Appendix 9 – Participant summary of results 205
Appendix 10 – Invitation to participate 206
Appendix 11 Questionnaire development 208
Appendix 12 – Participant questionnaire 211
Appendix 13 – Questionnaire for retrospective patients 220
Appendix 14 – Chapter four data frequency tables 229
Appendix 15 – Free text comments 237
Appendix 16 – PISCF Brushings study 240

List of Figures

Figure 2.1: Flowchart of participant recruitment and specimen testing. 38
Figure 2.2: p16 staining patterns. 47
Figure 2.3: qPCR figure panel. 48
Figure 3.1: Flowchart of study cases and testing performed. 65
Figure 3.2: Staining patterns of four specimens with focally positive p16 results. 72
Figure 3.3: CK19 immunohistochemistry image panel. 75
Figure 3.4: Viral load boxplots and scatterplot. 77
Figure 4.1: Flowchart of participant recruitment and analyses performed. 90
Figure 5.1: Flowchart of participant recruitment and testing performed. 125
Figure 5.2: Boxplots of viral loads from brushing samples. 131
Figure 5.3: Images of abnormal cytology. 134

List of Tables

Table 1.1: HPV related diseases and types associated. 7
Table 2.1: Primer sequences and PCR conditions. 41
Table 2.2: Comparison of demographic factors of the study population and national data. 46
Table 2.3: p16 result, and detection of a high risk HPV type by PCR/sequencing in archived oropharyngeal cancer biopsy samples. 49
Table 2.4: Univariable and multivariable analysis of putative risk factors associated with having an HPV positive tumour. 51
Table 3.1: Staining characteristics and HPV 16 DNA, RNA, Viral load, and CK19 results for p16 focally positive specimens. 73
Table 3.2: Sensitivities and specificities of p16 and CK19 compared to the gold standard of E7 mRNA detection. 76
Table 3.3: Kappa statistics, and 95% confidence intervals showing agreement between tests. 79
Table 3.4: Comparison of CK19 H-scores and HPV 16 E7 mRNA and DNA detection. 79
Table 4.1: Demographic characteristics of 89 patients with oropharyngeal cancer. 96
Table 4.2: Comparison of the demographics and occupations of the study population (n=89) and the NZWS (n=3003) 98
Table 4.3: Comparison of exposures to hazardous substances between study population (n=89) and New Zealand working survey (n=3003) with 95% confidence intervals for differences and exposures significantly higher in the study population than New Zealand working survey shown by shading. 99
Table 4.4: Description of alcohol, tobacco, and marijuana use of 89 oropharyngeal cancer patients. 101
Table 4.5: Description of sexual behaviours and sexual health of 89 oropharyngeal cancer patients. 104
Table 4.6: Summary of medical histories of 89 oropharyngeal cancer patients. 106
Table 4.7: Description of symptoms experienced by study participants in the six months prior to their diagnosis. 109
Table 4.8: Univariate analysis of putative risk factors associated ($p \leq 0.2$) with having a p16 positive tumour. 111
Table 4.9: Multivariable analysis (n=80) of putative risk factors associated with having a p16 positive tumour. 112
Table 5.1: Cytology results by tumour or non-tumour site. 133

Chapter 1 - Review of the literature

This literature review provides an overview of Human papillomavirus (HPV) and its role in human disease. The coverage of viral characteristics and the role of HPV in benign conditions are not intended to be exhaustive, but rather to provide background and context. As much of our knowledge of HPV is from cervical cancer studies, there is a substantial amount of cervical literature included. This is required as the knowledge and current practises used in the cervical setting provide a baseline and framework to build our knowledge of HPV in other cancers, including appropriate methods for laboratory diagnosis and possible disease prevention strategies. The major focus of this review is HPV in oropharyngeal cancer, including risk factors, laboratory diagnosis, and possible methods of disease prevention. Current data on the role of HPV in oropharyngeal cancer in New Zealand is also summarised.

1.1 Introduction

Human papillomaviruses belong to the genera *Papillomavirus* within the family *Papovaviridae* [1] . There are over 200 known types of HPV [2]. HPV are categorised in the alpha, beta, gamma, mu, and nu papillomavirus genera and include cutaneous and mucosal viruses [3]. Different HPV types are referred to as genotypes and are classified based on the L1 gene DNA sequence. A distinct type has an L1 DNA sequence at least 10% different from any other HPV type [3]. Mucosal HPVs are divided into high and low risk types based on their ability to cause cancer. There are 15 known high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and low risk types include types 6 and 11 [4].

HPV is highly transmissible and infection is very common [1,3]. There are two major modes of acquisition: casual contact, as is seen with cutaneous warts, and sexual contact seen with the mucosal types affecting the anogenital mucosa [5]. HPVs are of particular significance to human health as they are causative agents of a number of benign conditions, and more importantly, a number of cancers [6,7].

1.2 Characteristics of human papillomavirus

Papillomaviruses have non-enveloped icosahedral capsids measuring around 55 nm that are composed of only protein [8]. The viral genome consists of a circular double-stranded DNA molecule of approximately 8,000 base pairs [1].

1.2.1 Genome

The papillomavirus genome is divided into an early region (E), encoding various genes (E1-E7) that are expressed immediately after initial infection of a host cell, and a late region (L) encoding the capsid genes L1 and L2 [7]. All the genes are encoded on one DNA strand [8].

1.2.1.1 Early gene expression

The E1 and E2 genes are the only viral genes required for replication. The E1 protein binds to the viral origin of replication and assembles as a hexameric helicase facilitated by the E2 protein [7,9]. Viral DNA strands are then unwound and the viral genome replicated by host DNA replication factors [10]. The E2 protein also acts as a negative regulator of E6/E7 gene expression which is significant in viral integration and carcinogenesis [7,9].

The E4 protein is expressed at low levels early in viral infection but expression is highest in the late phase of infection. The E4 protein is understood to enable virion release [9].

E5, E6 and E7 are the transforming proteins of HPV. E5 is a small protein that destabilises membrane proteins in infected cells [9]. E5 has some transforming activities and interacts with Epidermal Growth Factor Receptor (EGFR), an important receptor in the oncogenesis of many cancers [7]. Its transforming abilities are less significant than those of E6 and E7 which are described below.

1.2.1.2 Late gene expression

The L1 protein forms the entire outer surface of the complete virion and can spontaneously assemble into virus like particles (VLP's) [11]. This knowledge has been exploited to create vaccines against HPV [12]. The L2 protein has a role in the infectious entry into cells and is able to interact with a number of host cell proteins [13].

1.2.1.3 Viral oncogene expression

Only the E6 and E7 proteins of high risk types are capable of transforming cells as the low risk proteins are unable to bind with enough affinity to cause any transforming actions within the cell [14]. The E7 protein is capable of transforming cells alone [7,9], however the effects of the two proteins are synergistic, and result in a lack of cell cycle control and unscheduled cellular proliferation [7]. The continued expression of the E6 and E7 genes are required to maintain the malignant phenotype [14]. It must be noted that although the E6/E7 proteins can initiate carcinogenesis, additional mutations in the host DNA are required for malignant progression to occur [14].

The major activity of the E6 protein is the inactivation of the tumour suppressor protein p53 [7,15]. Activated p53 acts to repair damaged DNA, arrest the cell cycle or induce apoptosis therefore preventing a build-up of mutations and genomic instability [15]. In addition, the E6 protein has other transforming abilities including telomerase activation, blocking apoptosis, disrupting cellular adhesion, polarity and differentiation and the ability to reduce recognition by the hosts immune system [15].

The E7 protein inactivates the retinoblastoma tumour suppressor protein (pRb) pathway through the breakdown of the E2F1-RB1 complex, resulting in activation of E2F1 regulated S-Phase genes [9,16]. This also leads to over expression of p16 (covered in section 1.6.3). The breakdown leads to aberrant S-phase entry and therefore uncontrolled cellular proliferation [14].

1.2.2 Lifecycle

Papillomaviruses infect squamous epithelium and their lifecycle is closely connected to the differentiation of the host cell. Infection can only be initiated in the undifferentiated basal cells, whereas replication can only be completed in the upper mature layers of the epithelium [1,17]. This link enables viral persistence and transmission by escaping many host immune mechanisms [18].

Papillomaviruses gain entry to the basal cells through micro-abrasions in either the skin or mucosal surface [9]. Viral entry is by an endocytic pathway mediated by the binding of viral L1 to cells [1]. L2 then facilitates the release of viral genomes into the cytosol [1].

The initial establishment phase of infection involves the virus replicating more frequently than the host cell in order to increase the viral copy number [1,6]. The following maintenance phase sees the virus replicating once per cycle in order to maintain the copy number in each daughter cell [1]. The productive phase of infection occurs in the outer layers of epithelium, where there is a dramatic increase in the copy number of virus and thousands of progeny viral particles are produced, leading to the formation of koilocytes. Cells containing the progeny virus are then desquamated allowing transmission [1,18].

1.3 HPV in benign conditions

Various HPV types are responsible for a number of benign and malignant conditions. A summary of conditions and the HPV types associated is shown in Table 1.1.

1.3.1 Cutaneous warts

Cutaneous warts caused by HPV are variable in appearance and may be colourless or pigmented, exo- or endo- phytic, and may occur singularly or be multifocal in distribution [5]. Each cutaneous HPV has a specific tropism for a particular body site, for example HPV 1 the soles of the feet, and HPV2 and 7 the hands [1].

Cutaneous warts are common and affect around 10% of the population [19]. They are rare before age five and peak incidence is in 10 to 14 year olds [8], with a lower incidence in older adults [5]. Generally warts are present for several months and in two thirds of cases they spontaneously regress within two years, although some can persist for longer [20].

Table 1.1: HPV related diseases and types associated.

Disease	HPV types associated
Common warts	2, 7
Plantar warts	1, 4
Flat cutaneous warts	3, 10
Epidermodysplasia verruciformis	Over 15 types, including types 5 & 8
Focal Epithelial hyperplasia (oral)	13, 32
Oral papilloma's	6, 7, 11, 16, 32
Anogenital warts	6, 11, 42, 43, 44, 55 and others
Anogenital malignancies	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66
Oropharyngeal cancer	16, 18 and anecdotal evidence for others

1.3.2 Focal epithelial hyperplasia (Heck's disease)

Heck's disease is caused by HPV types 13 and 32 and is characterised by numerous painless papules on the lips, tongue, and buccal mucosa [21]. Heck's disease is usually seen in children or adolescents [22]. Heck's disease has been described predominantly in people of the Eskimo, American Indian, and Latin American ethnicity, and is strongly associated with poverty and poor living conditions [22].

1.3.3 Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV) is a rare lifelong skin disorder associated with a high risk of skin carcinoma [23]. EV is an autosomal recessive disorder resulting in an abnormal susceptibility to many beta-papillomavirus types that are harmless to the general population [23]. EV lesions appear in childhood and present as disseminated flat reddish brown papules [5]. Around one third of patients develop squamous cell carcinoma of the skin with the average time between the appearance of benign lesions and development of cancer being 24 years [24]. The resulting skin cancers are most common in sun exposed areas and it is thought radiation has a role in carcinogenesis [6].

1.3.4 Genital warts

Genital warts are the most easily recognized sign of genital HPV infection and over 90% are caused by HPV types 6 and 11 [5]. Genital warts are sexually transmitted and highly infectious with over 65% of those with an infected partner developing genital warts within eight months [25]. The incidence of genital warts peaks between the ages of 20 to 30 years and males and females are equally affected [25].

1.4 HPV associated cancers

Persistent infection with a high risk HPV type can lead to the development of cancer. This process is mediated by the interactions of the viral proteins E6 and E7 [7]. These interactions result in induction of DNA synthesis, metabolic alterations, genomic instability, over-riding cell cycle checkpoints, resistance of apoptosis, and alterations in

the cytoskeleton or cellular polarity [14]. The most well studied of HPV related cancers is cervical cancer.

1.4.1 Cervical cancer

Cervical cancer is the second most common cancer in women worldwide with over 500,000 cases annually [26]. Over 80% of these cases occur in developing countries without formal screening programmes [26]. In the late 1970s, Dr Harold zur Hausen hypothesized cervical cancer was mainly caused by HPV [27]. Since then it has been established that HPV is the necessary cause for cervical cancer. A study by the International Agency for Research on Cancer (IARC) of 1000 cervical cancer cases from 22 countries, found HPV DNA in 99.7% of cervical tumours [6]. HPV types 16 and 18 are responsible for 70% of cervical cancers worldwide [28]. The next most frequently detected types are: 45, 31, 33, 52, 58, and 35 [6]. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 are recognised as known carcinogens of the cervix [29] while types 26, 68, 73 and 82 have been associated with cervical cancer in some studies and are considered to be possibly carcinogenic [6].

Anogenital HPV’s are sexually transmitted and sexual behaviours represent the major risk factors for HPV infection, and HPV related anogenital cancers [30]. Links between cervical cancer and sexual behaviours date back to 1842 when Antonio Rigoni-Stern reported higher rates of cervical cancer in married women and widows, than in virgins and nuns [27]. High-risk sexual behaviours such as an early engagement in sexual activity and an increased number of sexual partners are correlated with HPV associated warts, precancerous lesions, and cancer [14]. Although HPV is the necessary cause of cervical cancer, it is not a sufficient cause and a number of co-factors are

thought to be involved in the development of malignancy. The most widely accepted of these include tobacco smoking and co-infection with Human Immunodeficiency virus (HIV) or *Chlamydia trachomatis* [6,9]. The long term use of hormonal contraceptives, high parity, nutritional deficiencies, and infection with Herpes Simplex virus type 2 (HSV-2), or *Trichomonas vaginalis* have also been described, although their role in the development of malignancy is uncertain [28].

Genital HPV infection is very common with infection normally acquired soon after the commencement of sexual activity [31]. It is estimated that nearly all sexually active adults will be infected at some point in their lives [14,32]. The point prevalence of cervical HPV infection detected by PCR in cervico-vaginal specimens from females aged 14-59 was found to be 42.5% [31]. Cervical HPV infection peaks around age 20-24 and is less common in older women [31]. The majority of HPV infections are transient and cleared by cell mediated immune mechanisms [9].

The site of HPV infection is significant in cervical cancer and precancerous lesions as the vast majoirty of lesions arise from the transformation zone. This is an area where the endocervical columnar epithelium is replaced by metaplastic squamous epithelium [33]. This area is more sensitive to both HPV infection and the transforming actions of HPV [17]. In the transformation zone, HPV cannot complete its lifecycle resulting in an increased expression of HPV E6 and E7 and deregulation of growth control ultimately leading to the development of cancer [17]. An important step in cervical carcinogenesis is viral integration [14]. Integration results in an increased expression of

the viral E6 and E7 genes, as linearization usually interrupts the viral E2 gene, thereby eliminating its regulatory control of E6/E7 gene expression [9].

1.4.2 Other anogenital cancers

HPV is also causally associated with squamous cell carcinomas of other anogenital sites including the vagina, vulva, penis, and anus [6]. Cancers at these anogenital sites are less common than cancer of the cervix, although an increase in incidence of anal and vulvar cancers has been reported [34].

It is estimated there are 27,000 anal, 13,000 vaginal, 27,000 vulvar, and 22,000 penile cancers diagnosed worldwide each year [34]. HPV DNA is detected in 61-94% of vaginal cancers [6], and around 50% of penile cancers [34]. HPV associated vulvar and anal cancers are most commonly seen in younger patients, and the tumour has a basaloid appearance histologically [6]. HPV DNA can be found in up to 90% of these cases, but less than 10% of cases seen in older patients with tumours with a keratinising appearance [6].

Cancers that develop at these anogenital sites have defined precursor lesions, as seen in cervical cancer [14]. Anal cancer also arises from a transformation zone where squamous and columnar epithelia meet [14]. The risk factors for anal cancer are similar to those for cervical cancer and include HIV infection, smoking, practising anal sex, and the number of lifetime sexual partners [26]. As with cervical cancer, HPV 16 is the most commonly detected type in all anogenital cancers [26].

1.4.3 Head and neck cancer

Head and neck squamous cell carcinomas (HNSCC) comprise cancers of the oral cavity, larynx, hypopharynx, and oropharynx [35]. Historically, the majority of these cancers were seen in the larynx and oral cavity and were associated with smoking and heavy alcohol consumption [36]. However, since the 1960s, an association between HPV and HNSCC has been reported. These early reports arose from visual similarities between genital condylomata and laryngeal papilloma's [37]. The detection of HPV antigens in HNSCC by immunohistochemistry was described in the 1970s [38] and HPV 16 was first detected by polymerase chain reaction in three oropharyngeal cancer samples in 1985 [39]. Since then, the body of evidence has grown steadily. In 2005, the International Agency for Research on Cancer (IARC) concluded there was evidence for the causality of HPV 16 in squamous cell carcinomas of the oropharynx and oral cavity. There was limited evidence for HPV 18 and not enough evidence for other HPV types and other anatomical sites [6]. HPV related oropharyngeal cancer forms the focus of this thesis and as such will be covered in more detail below.

1.5 Oropharyngeal cancer

The incidence of HNSCC has decreased in parallel with a decrease in the number of individuals that smoke [40,41]. However, the incidence of oropharyngeal cancers (OPC) has increased in many developed countries [42]. The oropharynx includes the tonsils, base of tongue and oropharynx [43]. IARC estimates around 26% of OPC worldwide are HPV associated which amounts to 22,000 cases [43]. The proportion of cases reported to be due to HPV varies considerably between publications and the variation is

dependent on the population studied, location of the tumour, and method of HPV detection [44]. The proportion of OPC attributable to HPV in developed countries is likely to be much higher than the global estimate of 26% [43]. For example in 2006-07 93% of OPC cases in Sweden were HPV positive [45], and during 2000-04 in the United States 72% of OPC cases were HPV positive [46]. Over 90% of HPV positive OPC is due to HPV 16 [43]. Additionally, types 18 and 33 have been detected by a number of studies [44,47].

HPV positive head and neck cancers generally present as a small primary tumour, often with neck metastases. Some primary tumours are occult and their presentation is as a metastasis to a neck node. Tumours have a basaloid, non-keratinising morphology, lobular growth pattern, and are often infiltrated by lymphocytes [40,48]. Importantly, patients with HPV positive tumours show an improved prognosis, and an increased responsiveness to chemo- and radio- therapy [49]. The reasons for this increased responsiveness to treatment are thought to be due to the lower rates of genomic damage seen in HPV positive tumours [50]. The molecular profile of HPV positive and negative tumours is different with HPV negative cases more often having p53 mutations, gross deletions, and allelic losses [50,51]. However, a history of tobacco smoking can impact on patient prognosis with patients with a smoking history of ten or more packs years shown to have an increased risk of disease progression and death [52]. The improved prognosis of HPV positive OPC has led to studies around the de-intensification of treatments [53]. Current treatment regimens such as intensified radiation and concurrent chemoradiotherapy result in significant toxicities and long term morbidities including: severe dysphagia, dependence on a feeding tube, facial disfigurement, speech difficulties and changes in salivary output, pH and viscosity [54-

56]. The goal of de-intensification is to maintain current cure rates while minimizing long term morbidities [53]. Changing current treatments requires careful consideration and is not an option for those HPV positive cancers with a poorer prognosis such as those seen in heavy smokers and those with advanced disease [53]. Patient consultation is important and a study of 55 post-treatment oropharyngeal cancer patients found 69% of patients would chose more intense treatment (concurrent chemoradiotherapy over radiation alone) for a 5% increase in the given survival rate [57].

1.5.1 Risk factors for HPV positive oropharyngeal cancer

The most well studied risk factor for HPV positive oropharyngeal cancer is sexual behaviour. Transmission of oral HPV is primarily due to oral sex, however, salivary transmission through deep kissing has been reported [50,58]. The association with oral sex is further supported by the very low incidence of HPV positive OPC in African countries, where the practice of oral sex, particularly male oral contact with female genitals is uncommon [59,60].

It is thought the increasing incidence of HPV positive OPC seen in developed countries is due to changing sexual behaviours [42,61]. These changes have occurred over generations as a result of the increased availability of contraception, sexual expression in the media, and rebellion of the younger generation [62]. This idea is supported by data from the US, Australia, and Sweden which have shown an increase in the occurrence of premarital sex, oral sex, the number of lifetime partners, and a reduction in the age of sexual debut in recent birth cohorts [49].

The number of lifetime sexual partners is consistently associated with HPV positive tumours [61,63]. In a pooled analysis of eight case-control studies, HPV positive cancers of the oropharynx were associated with six or more lifetime sexual partners, and four or more lifetime oral sex partners [64]. Not surprisingly, other measures of high risk sexual behaviour including a young age at debut, oral-anal contact, having casual sex, having had a sexually transmitted infection, infrequent condom use and HSV-2 seropositivity have also been associated with an increased risk of HPV positive OPC [61,63-65].

HPV positive OPC patients are consistently found to be younger than their HPV negative counterparts [40]. Generally HPV negative cancers occur in the seventh decade of life [50] while the HPV positive cases are on average three to five years younger [50]. There is some confounding between the younger age of patients and sexual practices as Smith *et al* found those aged under 55 were more likely to have engaged in risky sexual practices and a significant difference in HPV detection was observed between the age groups, with 36% of younger cases HPV positive compared to 13% of older cases [61].

Tobacco use and alcohol have strong associations with HPV negative head and neck cancer but their associations with HPV positive cases are inconsistent, and when looking at the modifying effects of tobacco and alcohol on OPC reported interactions range from no interaction to super-additive interactions [50,66]. However, when limiting the reported data to only studies of oropharyngeal cancer as opposed to all head and neck cancers no associations between alcohol and/or tobacco and HPV positive OPC have been found [61,63,67,68].

Marijuana use has been found to be associated with HPV positive head and neck cancer. Gillison *et al* found the association increased with increased joint years (joint year: one joint per day for one year) and duration of use and persisted even when controlled for tobacco use [63]. Further evidence for the association was found in a pooled analysis of nine case control studies, where when adjusted for tobacco and alcohol use, ever having used marijuana was associated with an increased risk of oropharyngeal cancer without considering HPV status [69]. Although the carcinogenicity of marijuana in humans is uncertain [70], it is plausible it may affect the course of an HPV infection. Cannabinoids can alter immune responses in humans [71], and therefore may promote the initial HPV infection and/or viral persistence [63]. Interestingly the sexual revolution and increases in marijuana use occurred around the same time, and links to HPV positive OPC were described around a decade later. It is possible the full effects of marijuana use on OPC could be greater than currently described.

Diet and nutrition have been suggested to have associations with positive HPV status. In a study of 143 head and neck cancer patients, Arthur *et al* found positive associations between the pre-treatment intake of vitamin A, vitamin E, iron, β-carotene, and folate and HPV status. These results were corrected for age, sex, body mass index, tumour site, cancer stage, and problem drinking and/or smoking [72]. These results may be explained by the fact that HPV positive patients tend to be of higher socioeconomic status with regards to factors such as education, income, and oral health [73].

It has been suggested that changes in the frequency of tonsillectomies in the young have influenced the increase of HPV positive oropharyngeal cancers [74]. The tonsillar crypt is the likely site of HPV infection and therefore the removal of this tissue may be protective against future tonsillar cancer [75]. This observation does not however explain the increase seen in HPV positive base of tongue cancers over the same time period [74].

In cervical cancer there are many accepted co-factors that influence both viral persistence and progression. It is possible such co-factors exist for HPV positive OPC but these are yet to be identified. Possible directions for research include the presence of other medical conditions, medication use, and occupational exposures.

1.5.2 Development of HPV positive oropharyngeal cancer

The pathogenesis of HPV positive OPC likely has similarities with cervical cancer. It is assumed there is a long time between initial infection and the development of cancer, although this assumption is based on very limited evidence of temporality [66,76]. Evidence for this long lag time is from a study of 638 HNSCC patients and 1599 controls, all of whom had stored blood samples available [77]. These blood samples were taken with a median time before diagnosis of 6.3 years, and a maximum of 13.7 years. This study found HPV 16 E6 antibodies in the blood of 34.8% of oropharyngeal cancer patients, and 0.6% of controls. The study found no association between the detection of HPV 16 E6 antibodies and risk of oral cavity, laryngeal, or oesophageal cancer [77]. The major limitation of this study is the HPV status of the oropharyngeal tumours in the seropositive patients was unknown, and it was assumed based on smoking history and patient prognosis that these likely were HPV positive cases.

Without knowing the HPV status of the oropharyngeal tumour it is possible patients were seropositive due to HPV infection at another site. This is supported by the fact that a seropositive control subject developed anal cancer during the study period [77].

There are a number of differences between the development of cervical and HPV positive oropharyngeal cancers. Firstly, almost all cervical cancers arise from the transformation zone [33]. There is no equivalent site in the oropharynx although the tonsillar crypts are the likely site of infection [43]. Secondly, viral integration is an important event in cervical carcinogenesis however integration is reported to occur in less than half of OPC cases [78]. Another study reported only extrachromosomal forms of HPV 16 in 11 OPC cases [79]. It is believed a constant rather than high expression of viral genes E6 and E7 is required to drive carcinogenesis [80]. Thirdly, unlike cervical cancer, HPV positive OPC currently has no well-defined precancerous lesions [50], although areas of dysplasia adjacent to the tumour have been rarely reported [81].

Knowledge of the development of cervical cancer often uses studies of HPV detection in the cervix. However, publications on the natural history of HPV in the oropharynx have assumed that the oral detection of HPV can be used as a surrogate [36,66]. Although the detection of HPV in an oral rinse sample imparts an increased risk for the development of head and neck cancer [76], the sample is not taken from the infection site. Therefore knowledge from oral rinses assumed to apply to HPV in the oropharynx, such as a bimodal distribution in prevalence with age [82], and rates of infection being similar between the sexes [83] but quicker clearance in males [84], may not be applicable.

1.6 Laboratory Diagnosis of HPV

Strategies for the detection of HPV are varied in their design and detection targets which may be HPV DNA or RNA, viral oncoproteins or other surrogate markers [85,86]. HPV cannot be cultured in vitro [87] and serological methods are ineffective as only around 50% of HPV infected individuals produce detectable antibodies [5].

The usefulness of any HPV detection strategy relies not only on the ability to detect the presence of HPV, but also to discriminate between a transient infection and an active infection with the potential for malignant transformation [86]. Methods for the detection of HPV range from low sensitivity methods such as *in situ* hybridization, immunoperoxidase, and immunofluorescence [84,88] to moderately sensitive methods such as Southern blot, dot blot, reverse blot hybridization, and Hybrid Capture II [84,85,88]. Currently, the gold standard test is the detection of HPV E6/E7 mRNA as it reliably ascertains the presence of HPV and importantly, its biological relevance [47,86,89]. However, this approach is limited in clinical practice because of the requirement of a fresh specimen, when most diagnostic pathology laboratories work with formalin fixed paraffin embedded (FFPE) biopsy samples [86]. Polymerase chain reaction (PCR) detection of HPV DNA is the most sensitive method available [84]. The most common methods of HPV detection in research studies are PCR, In situ hybridisation (ISH), and p16 immunohistochemistry. Each method has associated advantages and disadvantages and these are described below.

1.6.1 Polymerase chain reaction

PCR can either use consensus primers to detect HPV DNA from a variety of HPV types, or type-specific primers for the detection of DNA from specific HPV types [4,85]. Other

approaches include the use of consensus primers followed by HPV specific probes for the detection of DNA from a range of high risk HPV types [44,88,89]. The type of tissue used (for example, formalin fixed or fresh) impacts the performance of PCR as the fixation process results in DNA fragmentation and often results in fragments of less than 200 base pairs [85]. Nonetheless, the major advantage of PCR is its high sensitivity [90] with some PCR methods able to detect as little as one HPV genome copy per cell [86], although the clinical relevance of such low levels are uncertain, and contamination must also be considered.

For HPV testing in cervical specimens there are a number of FDA approved commercially available assays often used in the diagnostic setting [91]. The test used reflects the facilities, resources and personal preferences of each laboratory [85]. In the investigation of cervical cancer, PCR based tests have a number of uses including triage of women with low grade or atypical smears, monitoring the success of treatment or a test of cure in those with previous high grade disease, or more recently a primary screening test (described below) [5]. The partial genotyping for HPV types 16 and 18 is common and triaged treatment referrals are often based on a combination of cytology and HPV 16/18 detection [91].

Conventional genotyping is time consuming and has a high cost [92]. Due to this other PCR based approaches have been developed by researchers in an attempt to provide a more timely way to detect and genotype HPV from samples. Dictor & Warrenholt developed a single-tube multiplex PCR for 21 HPV types which genotype samples using high resolution fluorescence capillary electrophoresis [4]. High resolution melt (HRM)

has also been used for genotyping, as described by Lee *et al* who detected eight high risk HPV types in an HRM assay [92].

In oropharyngeal cancer samples PCR is the most sensitive method for HPV detection. However, the simple detection of viral DNA does not allow for the assessment of viral activity or clinical relevance [93]. Use of a diagnostic algorithm combining PCR with another detection method helps to overcome this issue. A commonly used approach is to combine p16 and PCR. Schache *et al* used this approach and found that the sensitivity of PCR and p16/PCR was 97% compared to the gold standard of E6/E7 mRNA detection, but the specificity increased from 87% for PCR alone to 94% when using the algorithm [47]. Similar results were seen by Reitbergen *et al* when p16 protein expression was combined with PCR showing a sensitivity of 96% and specificity of 98% when compared to the gold standard [89].

1.6.2 In situ hybridisation

ISH is a signal amplification technique that employs labelled probes which are complementary to the target viral DNA sequence [86]. Similar to PCR, ISH can be used to detect many, or a single HPV type. Both HPV 16 specific and high risk HPV (to detect up to 13 high risk types) ISH are commonly used approaches [44,47].

ISH is described as more applicable than PCR to the diagnostic pathology setting due to the method being optimised for FFPE tissues [86] and is also able to localise HPV in the tumour cells [48]. Interpretation is visual with integrated HPV appearing as punctate nuclear staining, and episomal HPV as diffuse nuclear and cytoplasmic staining [85]. However, as previously described many HPV positive OPC contain only episomal HPV which may cause interpretative difficulties if only integrated HPV is reported as

positive. The visual interpretation of ISH can also be a disadvantage as inter-observer variability has been reported, with discrepancies in approximately 10% of cases [85].

ISH is commonly used in OPC research due to its ability to locate HPV within the tumour. However, the major disadvantage of ISH is its low sensitivity [85]. In a study comparing two commercially available ISH kits (Dako and Ventana) to the gold standard of mRNA detection, the sensitivity and specificity of the Dako kit was found to be 38% and 100% respectively, while the Ventana kit showed a sensitivity of 67% and specificity of 100% [94]. This difference between kits may be due to differences in the interpretation of results as true intra-nuclear staining was required for Dako specimens to be considered positive. This meant any specimen showing para-nuclear staining was considered HPV negative, this degree of nuclear localisation was not required using the Ventana kit. Results more supportive of ISH were found by Schache *et al* who used the same Ventana platform and tissue microarray specimen type as the above study, with both a sensitivity and specificity of 88% [47]. However, this was still below the reported values of sensitivity and specificity for both p16 protein expression and PCR methods.

1.6.3 p16 immunohistochemistry

p16 is a tumour suppressor protein that is a negative regulator of cell proliferation [85]. p16 overexpression is seen in active high risk HPV infection due to the inactivation of the retinoblastoma pathway by the HPV E7 protein [93]. Over-expression of the p16 protein is not specific for HPV, however, it is widely used in the laboratory setting as the method is accessible, simple and cost effective [85,86]. Interpretation of p16 IHC is based on the presence of brown staining within cells. A

positive p16 IHC staining result in lower anogenital tract specimens shows strong and diffuse block staining. This is further defined in the Lower Anogenital Squamous Terminology (LAST) guidelines as “continuous strong nuclear or nuclear plus cytoplasmic staining of the basal cell layer with extension upward involving at least one third of the epithelial thickness” [95].

The LAST guidelines recommend the diagnostic scenarios when p16 IHC should be used. These recommendations allow for diagnostic pathology reporting to correlate with current knowledge of HPV infections and the low grade /high grade approach to lesions without the inclusion of moderate dysplasia [96]. For example, in the cervical setting p16 IHC can be used in the following circumstances: to differentiate between high grade and mimic diagnoses, when moderate dysplasia (CIN2) is considered, to resolve professional disagreement, or in the case of colposcopy referral due to high risk HPV test results [96].

There is currently no accepted best practise for HPV detection in OPC samples [85]. Although usually used in combination with PCR and/or ISH in research [47,88,89,97], p16 is commonly used alone in the diagnostic setting [93]. This is supported by the reported sensitivity of p16 of 100% for HPV positive OPC in some studies [40,97,98]. The use of p16 alone may be problematic if used to provide prognostic information. It is reported that cases that are p16 negative but contain high risk HPV DNA are a distinct group with intermediate survival characteristics compared to p16 positive /DNA positive and p16negative / DNA negative cases [88,99]. Further difficulties with p16 providing prognostic information were shown in a study which reported no enhanced survival associated with either p16 or DNA detection alone, but only with the combination of positive results from two tests, either HPV DNA and E6/E7 serology

or p16 and E6/E7 positive serology [100]. These results suggest that to provide accurate information for patients a multi-test approach may be required.

The specificity of the p16 assay in HPV positive OPC is reported to be around 80% [40]. In addition, there is no direct or mechanical link between HPV and p16 which has a number of implications for testing. Firstly, p16 protein over-expression may be seen due to non-HPV related processes [86]. A small discrepancy of around 5% between the p16 assay and HPV status, determined by ISH was described in two studies [97,98]. In another study, this discrepancy has been described to be as high as 25% [40]. Secondly, it is important that the anatomical site is considered before applying the p16 assay. The oropharynx (in particular the tonsils) is preferentially targeted by HPV. Doxtader *et al* found of 25 p16 positive OPC samples, HPV DNA was detected in 24 (96%), while in other HNSCC five out of eight (63%) p16 positives contained HPV DNA. This decreased to one out of six (17%) of oesophageal samples [98]. p16 positivity has been shown in the majority of undifferentiated sinonasal carcinomas and around 60% of non-small cell lung carcinomas, although these are not associated with and do not contain HPV [93].

Unlike the LAST guidelines from the WHO used in genital p16 testing, there are no accepted guidelines for p16 in OPC. The arbitrary cut-off value of 70% strong and diffuse staining is used by many researchers [44,47,62,88,89]. However, others argue that a scoring system including the percentage of cells stained and the amount of confluent staining should be used [101,102]. In addition, it has been described that if a cut-off point of 75% of tumour cells showing strong, diffuse staining is used, 100% of

samples will express E6/E7 mRNA [94]. This approach may help improve the reported specificity of the test.

1.6.4 Other biomarkers

The search for biomarkers that reliably detect HPV and provide prognostic information is ongoing. Gene expression analysis has revealed groups of proteins which are over-expressed in HPV positive HNSCC compared to normal tissue and HPV-negative HNSCC [90,103,104]. The majority of these proteins are involved in DNA replication and cell cycle control [103]. Many of these proteins are regulated by E2F transcription factors and can be directly linked to the actions of HPV E6 and E7 [103,105] Other less predictable changes in expression have been described including three testis specific genes. Testicular cell adhesion molecule 1 (TCAM1), Synaptonemal complex protein 2 (SYCP2), and Stromal antigen 3 (STAG3) are consistently upregulated in HPV positive tumours [105,106] with 100,000-, 15-, and six- fold increases in expression respectively [106]. Patterns of viral gene expression such as high HPV E6, low E1^E4 fusion protein, and low or absent L1 have also been described for HNSCC [107].

1.7 Prevention of HPV associated cancers

Cancer prevention strategies include both primary and secondary prevention. Primary prevention aims to prevent persistent HPV infections, whereas secondary prevention aims to detect a precancerous stage and provide early curative treatment [108]. HPV related cancers, in particular cervical cancer, have well known primary and secondary prevention strategies in place internationally [1,108].

1.7.1 Primary prevention - Vaccination

The knowledge that the HPV L1 protein could spontaneously assemble into virus like particles (VLPs), which mimic the natural virus and elicit protective neutralising antibodies, facilitated the development of prophylactic vaccines [12]. Cervarix® and Gardasil® were initially the two vaccines approved for use, and in 2014 Gardasil®9 gained FDA approval. Cervarix® is a bivalent vaccine for types 16 and 18, while Gardasil®4 contains types 16, 18, 6 and 11 [36], and Gardasil®9 has an additional five high risk HPV types (31, 33, 45, 52, and 58) [109].

The cervical cancer vaccine is considered the most expensive vaccine ever produced with immunisation costing up to $360 USD per individual [32], although the costs of production are likely much lower. The vaccine is approved for use in males and females aged 9-26 years with immunisation initially requiring three doses over six months [110]. However, studies have shown non-inferior vaccine efficacy when only two doses are given [111,112]. Current guidelines for Gardasil®9 are for two injections six months apart in those aged up to 14 years, but three injections for those aged 15 years old or older [111]. These guidelines are currently used in New Zealand and reflect the stronger immune response elicited in younger individuals [12]. The target age for many Gardasil® immunisation schedules is young adolescents (around age 12) and this highlights the importance of vaccination prior to the onset of sexual activity, and therefore HPV exposure. The reduced number of injections has allowed an improved cost-effectiveness and as such some developed countries including Australia and New Zealand now include males and females in their funded vaccination schedules [113,114].

The biggest issue since the vaccine introduction has been the less than desired coverage rates. Coverage of 50% or more females vaccinated has demonstrated population-level effects including a 61% reduction in the HPV 16/18 infection rate between the pre- and post- vaccine periods [115]. Vaccine coverage was initially low, driven mainly by parental concerns due to the sexual nature of HPV infection and vaccine safety [32]. However, in recent years many developed countries are reporting higher coverage rates with the highest rates seen in northern Europe, Australia and New Zealand [116]. In terms of global uptake of the vaccine, 118 million females have been targeted by an HPV immunisation program and 1.7% of the global female population have received at least one dose, the majority of these being from middle or high income countries [116]. Therefore, populations with the highest burden of cervical cancer remain largely unprotected [110,116]. In recent years a number of global initiatives and demonstration programs including the vaccine alliance (Gavi), the Gardasil access program, and PATH have provided vaccines for developing countries [116]. The administration of even one dose of the vaccine per female in countries where the cervical cancer burden is the highest will likely have large impacts on both incidence and mortality in the future.

To date, Gardasil® has shown strong effectiveness in the prevention of genital warts. Australia has seen a 77% reduction in genital warts in vaccine eligible females aged 12-17 years [32]. Protection against HPV 16/18 related cervical intraepithelial neoplasia (CIN) was reported as 100% after five years in those receiving the vaccine [117]. Lower vaccine efficacies have been calculated in other population based studies. For example results from the United States National Health and Nutrition Examination Surveys (2003-10) showed a reduction in the prevalence of HPV types 6,11, 16 and 18 in 14 to

19 years olds from 11.5% (95% CI: 9.2-14.4) in 2003-06 (pre-vaccine era) to 5.1% (95% CI: 3.8-6.6) during 2007-10 (vaccine era) [118]. The prevalence in the vaccine era can be attributed to the low vaccine coverage seen in the United States.

1.7.1.1 Vaccination for HPV positive oropharyngeal cancer

It is biologically plausible that the vaccine may have a role in the prevention of HPV positive OPC. This may be through direct prevention of oropharyngeal infection, or by a reduction in the genital burden of HPV, thereby limiting the spread to the oropharynx [108]. Evidence supporting the vaccine preventing OPC comes from the vaccine's proven efficacy against anal intraepithelial neoplasia with a calculated vaccine efficacy of 77.5% in a group of 299 16 to 26 year old men who have sex with men [119]. This vaccine efficacy may be considered higher than expected given all study participants were sexually active at the time of enrolment. Further evidence is shown in the Costa Rica vaccine trials where a 93% reduction in the prevalence of oral HPV infection between the vaccine and control groups was found four years after vaccination. This figure is based on 15 infections in the control group and one in the vaccine group [120]. No oral rinse samples were taken at baseline, therefore, it is possible the infection in the vaccinated individual was present at the baseline, thus the vaccine efficacy may be higher than that reported by the study.

1.7.2 Secondary prevention - Screening

Although the majority of cervical HPV infections resolve, some progress and cause precancerous lesions or cancer [14]. Cervical precancerous lesions or CIN are well defined and show a continuum of cellular changes both histologically and cytologically [33]. Histologically, CIN is graded from CIN1 to CIN3 as these precancerous lesions

range in severity from mild to severe dysplasia [33]. There is a low grade/ high grade approach to the cytological reporting of cervical precancerous lesions. Low grade lesions (LSIL) are often transient infections whereas high grade lesions (HSIL) have a much higher potential to develop into invasive cervical cancer [17]. The development of CIN3 is thought to take 7-15 years, thus, CIN3 diagnosis rates peak in those aged 25-30 years [28]. If left untreated, CIN3 lesions may progress to cervical cancer, a process that is thought to take around 10 years [28]. The detection and subsequent treatment of these precancerous lesions forms the basis of cervical screening [33]. Cervical cytology has been widely used since it was first described by George Papanicolaou in the 1940s [27]. However, the test suffers from low sensitivity.

In recent years, the idea of replacing cytology with a PCR based test has gained support. The ATHENA trial of 40,901 women aged over 25 in the United States found primary HPV testing using PCR identified 64.2% more CIN3 or higher lesions than cytology [121] and that almost half of the CIN3 or higher cases identified during the three years of the study occurred in those with a baseline negative cytology result [121]. Similarly, the COMPASS trial pilot phase of 5006 women aged 25-64 in Australia found primary HPV screening was associated with a significantly higher high grade detection rate than cytology [122]. It is therefore likely many developed countries will change to HPV primary testing in the near future with countries such as Australia and the Netherlands already having made the switch [121,122].

1.7.2.1 Screening for HPV positive oropharyngeal cancer

Effective prevention through screening relies on the identification of disease at an early stage and effective treatment of the lesion resulting in reduced mortality from

the cancer [108], unfortunately, neither of these are known for HPV positive OPC. Based on similarities to cervical cancer, a 'pap-test equivalent' has been proposed for HPV positive OPC [108]. A study using a pap-test equivalent found an association between HPV 16 detection and oropharyngeal cancer with an odds ratio of 6.1 (95% CI, 1.6 – 22.7) in patients with visible oropharyngeal lesions. However, when the same test was applied to HIV-positive patients with no visible abnormalities, the detection of HPV 16 was not associated with cytological abnormality [123]. It must be noted this study population may be a limiting factor as they did not reflect OPC patients from the same country in terms of the distribution of age, gender or ethnicity [46]. It is also possible differences between the cervical and oropharyngeal anatomy may explain these results. The transformation zone of the cervix is relatively accessible whereas the tonsillar crypts are hidden and not accessible by any conventional sampling device such as a cytobrush [124]. HPV DNA has previously been detected in the tonsils from cancer cases and cancer free individuals by either brushings taken under general anaesthetic [123,125] or the use of ex vivo brushings [126,127]. Using anaesthetised patients limits the interpretation of how brushings could be applied as a screening test as the procedure imparts significant financial and time costs, and risks for the patient. To date there is little work on a minimally invasive sampling procedure on conscious patients. A further complication of screening for OPC is although the incidence is increasing, it is still low and the required number to screen to detect one case would not be cost effective [128]. Therefore, it is unlikely any population based screening tests will be introduced, and future applications may centre on earlier detection of smaller tumours, as opposed to precancerous lesions, with a focus on reduced treatment morbidities.

1.8 Oropharyngeal cancer in New Zealand

Currently, data on the contributions of various HPV types to OPC in New Zealand is limited [129,130]. The number of cases of oropharyngeal cancer caused by HPV in New Zealand is unknown, however, when looking at anatomical site alone Ministry of Health data shows there has been an increase in the number of oropharyngeal cancer cases from 35 cases in 1996 to 126 cases in 2012. In contrast, the incidence of oral cavity cancers has remained steady during the same time period [131].

It is likely New Zealand's cases follow international data with HPV 16 responsible for the vast majority of cases. In a study of 55 OPC cases from 2010-11 from the Auckland region, 76% (42/55) were p16 positive, and of these 98% (41/42) contained HPV DNA. HPV 16 was present in 88% of cases, there were three cases with HPV 33, and one each of HPV18, 35, and a 16/33 co infection [130].

The Ministry of Health reported 116 incident cases of squamous cell carcinoma of the cervix and 126 cases of oropharyngeal cancer in 2012. Assuming around 75% of the OPC are caused by HPV [130], it is likely the number of HPV positive OPC cases will soon equal the number of cervical cancer cases. The USA is estimated to reach this equilibrium by 2020 [23]. This situation and new gender balance in HPV related cancers highlights the importance of the expansion of the New Zealand vaccination schedule to include males, introduced in 2017.

1.9 Conclusion

This review has described the role of HPV in oropharyngeal cancer, with a focus on laboratory diagnosis, risk factors, the potential for disease prevention, and the situation in New Zealand. The following areas where there are gaps in our knowledge of this disease have been identified. Firstly, the magnitude of the burden of HPV positive OPC in New Zealand is currently unknown, and this must be ascertained in order to make evidence based decisions around vaccine delivery and to accurately calculate potential vaccine impacts. Secondly, current testing methods used in diagnostic laboratories fall short of the gold standard, which is not compatible with the specimen types used in histopathology laboratories. Thirdly, the known risk factors for HPV positive OPC are largely those related to the sexual transmission of the virus. Other possible co-factors such as occupational exposures and other medical conditions require investigation. Finally, although a pap-test equivalent has been suggested for OPC, it is currently unknown if a brush based test on conscious individuals with oropharyngeal cancer is capable of detecting HPV DNA or cytological abnormalities. Furthermore, such abnormalities or precancerous lesions are yet to be described for HPV positive OPC. Research into these areas will promote a better understanding of HPV positive OPC, which in turn will help ensure the best possible outcomes for future patients.

Chapter 2 - The prevalence of human papillomavirus in oropharyngeal cancer in a New Zealand population

Published PLoSONE article in appendix one

2.1 Abstract

The incidence of oropharyngeal cancer (OPC) in New Zealand (NZ) has more than doubled over the last 14 years with 126 cases in 2012. Overseas studies have shown that human papillomavirus (HPV) plays a significant role in the development of these cancers. However, the role of HPV in OPC and the burden on the NZ health system is unclear.

Aim: The aim of the study was to determine the prevalence and the genotypes of HPV associated with OPC in New Zealand.

Methods: In this study, 621 OPC were identified from cancer registry data from 1996-98, 2003-05, and 2010-12. Biopsies of 267 cases were then retrieved from laboratories throughout New Zealand. p16 immunohistochemistry and a human beta-globin PCR were performed on all specimens. HPV genotyping was performed on all beta globin positive specimens using real-time PCR with melt analysis.

Results: Using a p16/PCR algorithm, 77.9% (95% CI: 71.1 – 83.5%) of cases were attributable to HPV. Of these, 98.5% were HPV 16 positive. There was also one case each of HPV 33 and 35. The percentage of HPV positive cases increased from 61.9% (95% CI: 40.9% – 79.2%) in 1996-98 to 87.5% (95% CI: 79.8% – 92.5%) in 2010-12. Results from the multivariable model, adjusted for sex and ethnicity, found statistically significant associations between HPV positivity and timeframe (OR: 5.65, 95% CI: 2.60-12.30, 2010-12 vs 1996-98), and between HPV positivity and patient age (OR: 0.55, 95% CI: 0.33-0.99, ≥61 years vs ≤60 years).

Conclusions: This data is consistent with data from other developed countries showing an increase in cases of HPV positive OPC in New Zealand, and the majority of cases being attributable to HPV 16. These results support the recent inclusion of males into the nationally funded immunization schedule for Gardasil® 9.

2.2 Introduction

Head and neck squamous cell carcinomas (HNSCC) comprise cancers of the oral cavity, larynx, hypopharynx, and oropharynx [35]. The incidence of HNSCC in developed countries has decreased in parallel with a decrease in the number of individuals that smoke [40,49]. However, the incidence of oropharyngeal cancers (OPC) has increased [41]. The proportion of cases reported to be due to human papillomavirus (HPV) varies considerably between studies and the variation is dependent on the population studied, location of the tumour, and method of HPV detection [42]. HPV 16 is a recognised carcinogen in the oropharynx, while there is limited evidence for the role of HPV 18 in OPC, and insufficient evidence for other HPV types [6]. Worldwide over 90% of HPV positive OPC are due to high risk HPV 16 [43], with as many as 97% of cases reported by some studies to be attributable to HPV 16 [47,88]. Other high risk HPV types such as types 18, 31, and 33 have also been detected [43,44,47].

In the past, the majority of HNSCC was seen in the larynx and oral cavity and was associated with smoking and heavy alcohol consumption [41]. The increasing incidence of HPV positive cancer of the oropharynx (including tonsillar and base of tongue cancers) over the last three decades is thought to be due to changing sexual behaviours and this is represented in the risk factors for the disease [41,61,62]. Data

from the US, Australia, and Sweden has shown an increase in risk factors such as the occurrence of premarital sex, oral sex, the number of lifetime partners, and a reduction in the age of sexual debut in recent birth cohorts [50]. Other risk factors include marijuana use and diet and nutrition [63,72].

The number of cases of OPC caused by HPV in New Zealand is unknown, however, when looking at anatomical site alone there has been an approximately threefold increase in the number of OPC cases from 35 cases in 1996 to 126 cases in 2012 (Ministry of Health, 2014). In contrast, the incidence of oral cavity cancers has remained steady during the same time period [131]. Currently there is no published data on the contributions of various HPV types to OPC in New Zealand. It is likely New Zealand's cases follow international data with HPV 16 responsible for the vast majority of cases. Thus, this study aims to determine the prevalence and the genotypes of HPV associated with OPC in New Zealand.

2.3 Materials and methods

Ethics approval was obtained from the Health and Disabilities Ethics Committee (reference: 14/STH/128), the approval letter is provided in appendix 2. Participant privacy was preserved by de-identification of samples.

2.3.1 Participant recruitment and sample collection

A flow chart of participant recruitment and specimen testing is shown in Figure 2.1. Cases were identified by a New Zealand cancer registry search of all cases of oropharyngeal cancer of ICD-10 codes C01, C05.1-5.2, C09.0-9.9, and C10.0-10.9 [132]

from 1996-98, 2003-05, and 2010-12. The New Zealand cancer registry is a population based register of all primary malignant diseases diagnosed in New Zealand. The information available from the registry comprises the following: patient demographics (age, sex, and ethnicity) and tumour specific information (tumour site, morphology, grade, extent, and diagnosis date, laboratory code and basis for diagnosis (histology, cytology etc)). Data was cross checked with National Health Index (NHI) data to identify patients that were deceased. Study information sheets and consent forms (provided in appendix 3) were sent to all living patients whose samples were stored in laboratories with five or greater specimens in storage as per the laboratory code from the cancer registry data, and with current and complete address details. In addition the whānau (extended family) of deceased participants identified as Māori from the Auckland region were requested to give consent for these specimens to be used, as per local Iwi (tribe) wishes. All participants provided written informed consent prior to specimens being requested from storage. After specimen requests were made, laboratories sent the specimens (n = 267), a copy of the pathology report, and any existing p16 slides (n = 57) to Massey University.

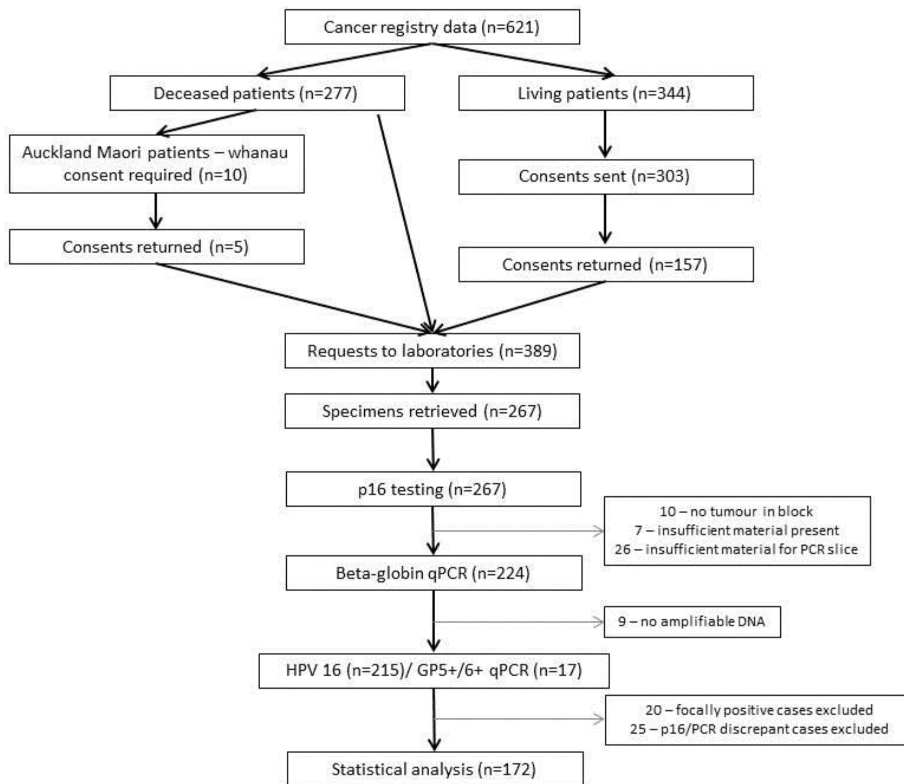

Figure 2.1: Flowchart of participant recruitment and specimen testing.

2.3.2 p16 IHC

Samples with a historic p16 slide had the existing slide used in this study. Samples were recoded and read blinded. All other samples had p16 IHC performed at a diagnostic medical laboratory (MedLab Central Ltd, Palmerston North, New Zealand). Formalin fixed paraffin embedded (FFPE) tissue was used for IHC. Sections were cut at four microns and baked onto Superfrost Plus slides at 60°C for 60 minutes. Staining was performed on the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, USA), with antigen retrieval performed on board, using Ventana CC1 buffer for 32 minutes at 100°C. A 1:150 dilution of the p16 (INK4a) antibody (G175-405, product code 551153) (BD Biosciences, North Ryde, Australia), was incubated on the slide for

eight minutes at 36°C. The detection of p16 was then visualised using the Ventana Optiview DAB kit (Ventana Medical Systems). A multi-tumour block positive control containing a serous ovarian carcinoma was included with each run of samples. All slides were read independently by two pathologists (BL and UV), and discordant cases reviewed to reach a consensus. Samples showing strong diffuse staining in the nucleus and cytoplasm of greater than 75% of tumour cells were considered positive. Samples showing staining in 10% to 75% of tumour cells were considered focally positive, and samples with staining in less than 10% of tumour cells were considered negative.

2.3.3 DNA extraction

A 10 µm slice of representative FFPE tumour block from each case with sufficient material (n=224) was cut for DNA extraction using a new blade between each sample to avoid cross contamination of samples. DNA extractions were performed using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) as per manufacturer's instructions for tissue. A control extraction containing only kit reagents and no sample was included with each set of samples. The blank extractions were then used as negative controls in the beta globin and HPV 16 qPCRs. A pre-treatment for paraffin embedded samples was included, and a final elution volume of 100 µL was used. The quality of extracted DNA was assessed using a Nanodrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

2.3.4 Beta-globin qPCR

The presence of amplifiable DNA was assessed on all cases with extracted DNA by qPCR targeting the human beta-globin gene using the PC03 and PC04 primers [133](Protocol shown in appendix 4). All primers used are shown in Table 2.1. Each

20µL reaction mix contained: 0.25 µM each primer, and 1X Fast Start SYBR Green Master (Roche Diagnostics, Basel, Switzerland). Up to 50 ng of extracted DNA was used as template. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles of: 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds. PCR was followed by a melt curve from 75 – 85°C with 0.2°C increments and a three second hold. A sample was considered positive if it produced a T_m of 81.0°C (±1.0°C). Samples showing low amplification on the qPCR were visualised using gel electrophoresis through a 1.0% agarose gel containing 1X SYBR® Safe (Thermo Fisher Scientific) to confirm product amplification.

Table 2.1: Primer sequences and PCR conditions.

Primer name	Primer sequence (5'-3')	Annealing temp	Gene target/ position	Product Size (bp)	Reference
16F cloning	GAT CAG TTT CCT TTA GGT CG	62 °C	HPV 16[1]	1775	Primers designed for this project
16R cloning	GGT ACC TGC AGG ATC AGC CAT		(7014-885bp)		
PC03	ACA CAA CTG TGT TCA CTA GC	55 °C	Human beta globin gene[2]	110	Saiki et al [133]
PC04	CAA CTT CAT CCA CGT TCA CC		(827-937bp)		
GP5+	TTT GTT ACT GTG GTA GAT ACT AC	49 °C	HPV L1 gene[1]	140	de Roda Husman et al [134]
GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C		(6624-6765bp)		
16F	GTG GAC CGG TCG ATG TAT GTC T	62 °C	HPV 16 E6[1]	209	Dictor and Warrenhalt [4]
16R	TCC GGT TCT GCT TGT CCA GC		(496-704bp)		

[1] Genbank accession number: KO2718

[2] Genbank accession number: L26478

2.3.5 HPV 16 qPCR

The presence of HPV 16 was assessed on all cases with a positive beta globin qPCR result. A qPCR with melt curve analysis (protocol shown in appendix 5) was developed using previously described primers [4]. Each reaction mix contained: 0.25 µM each primer, and 1X Fast Start SYBR Green Master (Roche Diagnostics). Up to 50 ng of extracted DNA was used as template. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles of: 95°C for 15 seconds, 62°C for 30 seconds and 72°C for 30 seconds. PCR was followed by a melt curve from 70 – 80°C with 0.2°C increments and a five second hold. A sample was considered positive if it produced a T_m of 77.6°C (±1.0°C).

A positive control was made from cloned DNA of a HPV 16 positive clinical sample provided by MedLab Central Ltd (Palmerston North, New Zealand). Cloning was performed using a pGEM®-T Easy kit with JM109 High Efficiency Competent Cells (Promega, Madison, USA), according to the manufacturer's instructions with blue/white selection. The cloned plasmid contained an approximately 1.8 kb fragment of the HPV 16 genome that included the E6 and E7 gene sequence amplified using the primers detailed in Table 1. Before being used as a positive control, the cloned plasmid was subjected to automatic dye-terminator cycle sequencing with BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyser (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) to confirm genomic sequence using both the forward and reverse primers. The sequences obtained were compared using the NCBI Blast database to other published sequences available from GenBank [135].

Duplicate standard curves from eight, 10-fold serial dilutions of plasmid in water, starting from 9.56 x 10^7 copies of HPV 16 were used to determine the limit of detection of the HPV 16 qPCR.

2.3.6 Confirmation of non-HPV 16 types

Samples that were p16 positive / focally positive, and HPV 16 negative were subject to qPCR using the GP5+/6+ primers [134] (protocol shown in appendix 6). Each reaction contained 0.2 µM each primer, 2.0 mM $MgCl_2$, 1X PCR buffer, 0.3 mM each dNTP, 1 unit Platinum™ *Taq* (Invitrogen, Carlsbad, USA), 1.5 µM Syto® 9 (Invitrogen). Up to 50 ng extracted DNA was used as template. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles of: 95°C for 15 seconds, 49°C for 30 seconds and 72°C for 30 seconds. PCR was followed by a melt curve from 70 – 85°C with 0.2°C increments and a five second hold. Known HPV 16, 18, 33, and 52 positive samples were included in each run. Samples with a T_m in the range of 76.0 – 81.0°C were considered positive for HPV DNA. The GP5+/6+ PCR could not reliably discriminate HPV genotypes therefore, the product of samples positive for the GP5+/6+ PCR were purified using the PureLink® PCR purification kit (Invitrogen, Carlsbad, CA, USA). The HPV type present was then confirmed by sequencing as previously described.

2.3.7 Data handling and statistical analysis

The continuous variable age was categorised into a binary variable: aged 60 years or younger, and 61 years or older. Ethnicity was classified into NZ European, NZ Māori, and other, with NZ Maori, then other prioritised.

To investigate the association between HPV positive OPC and putative risk or confounding factors (age, sex, ethnicity, and time period), each factor was tested

individually for significance at $p < 0.2$ in a logistic regression model using the software package R version 3.2.0 (R Development Core team, 2010, R Foundation for Statistical Computing, Vienna, Austria). A multivariable model was built by a stepwise selection process, retaining all variables significant at $p < 0.05$ and any confounding variables. Once a main effects model was built two-way interaction terms were introduced to the model and retained if significant at $p < 0.05$.

2.4 Results

2.4.1 Study population

The national dataset from the cancer registry comprised a total of 621 cases from the 1996-98 (n=113), 2003-05 (n=185), and 2010-12 (n=323) time periods. Diagnosis was based on histology in 94.8% (n=589) of cases, cytology in 4.6% (n=29) of cases and other non-specified tests in 0.6% (n=3) of cases. The proportion of cases diagnosed on histology versus cytology was consistent over all time frames. The mean age of an OPC patient was 59.2 years. NZ Europeans made up 68.8% of the national cases, and NZ Māori a further 10.5%. There was an approximate 4:1 ratio of males to females.

Of the 344 living patients identified from the national dataset, 303 were able to be contacted, of which 52% (157/303) gave consent. Of the 157 cases, 109 specimens could be retrieved. There were 277 deceased patients identified in the national dataset. Of the 277 cases, 158 specimens were retrieved for use in the study.

Thus, the total study population comprised 267 cases with retrievable specimens, and represented all major centres. Between four and 61 cases were retrieved from each of

the 15 laboratories involved. The demographic factors of the national dataset and study population were comparable as shown by the high p-values for age, sex, ethnicity and timeframe (Table 2.2). Therefore, the study population was considered to be reflective of the national dataset.

Table 2.2: Comparison of demographic factors of the study population and national data.

	National dataset (n=621)	Excluded cases (n=354)	Study population (n=267)	p-value[1]
Mean age at diagnosis	59.2 years	58.6 years	60.1 years	0.30
Sex : Male	492 (79.2%)	283 (79.9%)	209 (78.3%)	0.82
Female	129 (20.8%)	71 (20.1%)	58 (21.7%)	
Ethnicity: NZ European	427 (68.8%)	237 (66.9%)	190 (71.2%)	0.77
NZ Māori	65 (10.5%)	36 (10.2%)	29 (10.9%)	
Other	118 (19.0%)	73 (20.6%)	45 (16.9%)	
Not stated	11 (1.7%)	8 (2.3%)	3 (1.0%)	
Timeframe: 1996-98	113 (18.2%)	62 (17.5%)	51 (19.1%)	0.83
2003-05	185 (29.8%)	102 (28.8%)	83 (31.1%)	
2010-12	323 (52.0%)	190 (53.7%)	133 (49.8%)	
Deceased	277 (44.6%)	119 (33.6%)	158 (59.2%)	<0.01
Living	344 (55.4%)	235 (66.4%)	109 (40.8%)	

[1] p-value calculated compares the study population to the national dataset.

2.4.2 p16 IHC

p16 results were categorised as positive, focally positive, or negative (Fig 2.2). Consensus was achieved in all cases. A positive result was seen in 58.4% (156/267) of cases, 7.9% (21/267) were focally positive, and 27.3% (73/267) were negative. The remaining 6.4% (17/267) of cases had no tumour present in the block (n=10), or had insufficient material present (n=7).

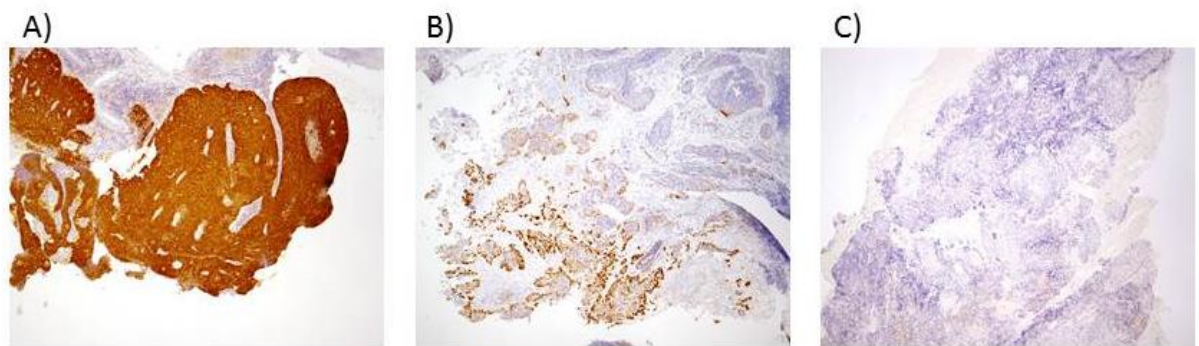

Figure 2.2: p16 staining patterns. A) Positive sample with >75% of tumour cells showing strong diffuse staining (magnification 40 x) B) Focally positive samples with 10 - 75% of tumour cells stained (magnification 40 x), and C) a negative sample with <10% of tumour cells stained (magnification 40 x).

There were 57 cases with a historic p16 slide. When comparing study p16 results to original results from cases with historic p16 slides, concordance was seen in 49/49 positives, 1/2 focally positives, and 5/5 negatives. The discordant focally positive case was identified as negative by our study. The remaining case had a slide sent for the study but no p16 result in the original pathology report.

2.4.3 Beta-globin, HPV 16, and GP5+/6+ qPCR

DNA could be extracted from 224 samples. Following the beta-globin qPCR (Fig 2.3) a further 4.0% (9/224) were excluded due to a lack of amplifiable DNA. HPV16 qPCR was performed on all beta globin positive samples. Serial dilutions of a HPV 16 clone control showed the assay was capable of detecting 26 copies of the HPV 16 E6 target sequence (Fig 2.2). HPV 16 was detected in 74.4% (160/215) of cases.

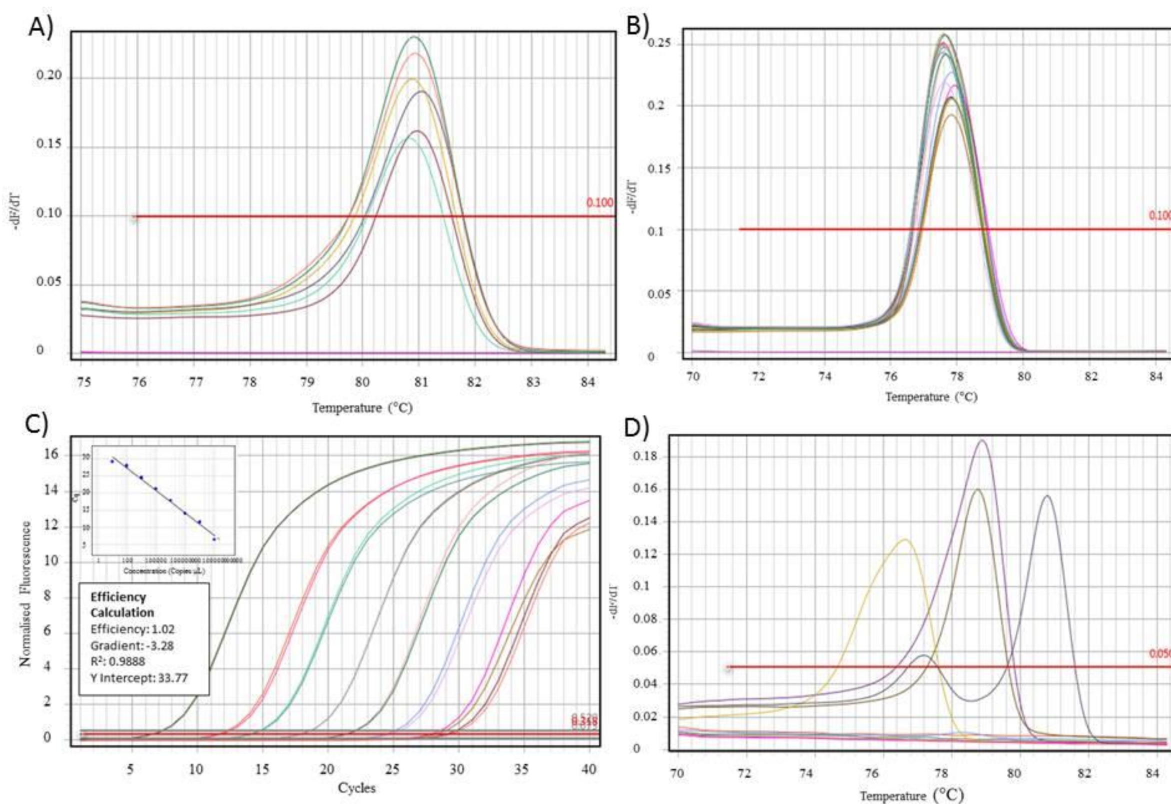

Figure 2.3: qPCR figure panel. A) Melt curve of beta-globin qPCR. Positive samples shown with T_m of 81.0 °C (±1.0 °C). B) Melt curve of HPV 16 qPCR. Positive samples shown with T_m of 77.6 °C (±1.0 °C). C) Standard curve of HPV 16 clone control serial dilutions. D) Melt curve of GP5+/6+ qPCR. Positive samples shown with T_m in the range of 76.0 – 81.0 °C.

Results of the GP5+/6+ qPCR (n = 17) were 82% (14/17) negative, and 18% (3/17) positive (Figure 2.3). Of the three positive samples, HPV was confirmed by sequencing in two cases, one case each of HPV 33 and 35. The HPV type could not be detected in the remaining case. Overall, high risk HPV was detected in 75.3% (162/215) of cases. The detection of a high risk HPV type by PCR/sequencing and the p16 result is shown in Table 2.3.

Table 2.3: p16 result, and detection of a high risk HPV type by PCR/sequencing in archived oropharyngeal cancer biopsy samples.

		High risk HPV		Totals
		Positive	Negative	
p16 result	Positive	134	6	140
	Focally positive	11	9	20
	Negative	17	37	54
	Totals	162	52	214

For the purpose of further analysis, a sample is considered positive if positive by p16, and positive for either the HPV 16 qPCR, or a high risk HPV type was confirmed by sequencing. A negative sample is negative by p16, and HPV 16 qPCR negative. This approach resulted in an overall study prevalence of HPV positive OPC of 77.9% (95% CI: 71.1 – 83.5%). HPV 16 accounted for 98.5% of HPV positive OPC.

2.4.4 Statistical analysis

The proportion of cases attributable to HPV increased from 61.9% (95% CI: 40.9% – 79.2%) in 1996-98 to 63.8% (95% CI: 49.5% – 76.0%) in 2003-05, and finally to 87.5% (95% CI: 79.8% – 92.5%) in 2010-12.

The mean age of an HPV negative patient was 66.2 years (95% CI: 65.8 – 66.4), compared to 56.8 years (95% CI: 56.6 – 56.9) for an HPV positive patient. The univariate analysis of putative risk factors showed age ($p < 0.01$) and timeframe ($p < 0.01$) to be significant (Table 2.4). In the final multivariable model (Table 2.4) there was a statistically significant association between HPV positivity and time frame (OR: 5.65, 95% CI: 2.60-12.30, 2010-12 vs 1996-98). A statistically significant association between HPV positivity and patient age was also found (OR: 0.55, 95% CI: 0.33-0.99, ≥61 years vs ≤60 years). These estimates were adjusted for sex and ethnicity.

Table 2.4: Univariable and multivariable analysis of putative risk factors associated with having an HPV positive tumour.

		Univariable analysis[1]			Multivariable analysis (n=249)		
Variable		**Odds Ratio**	**95% CI**	**P value**	**Odds Ratio**	**95% CI**	**P value**
Age	≤60	REF[2]			REF[2]		
	≥61	0.48	0.28-0.82	<0.01	0.55	0.33-0.99	0.05
Sex	Female	REF[2]			REF[2]		
	Male	1.50	0.79-2.78	0.21	1.36	0.68-2.66	0.38
Ethnicity	NZ European	REF[2]			REF[2]		
	NZ Māori	1.82	0.74-5.15	0.22	1.49	0.55-4.52	0.45
	Other	1.07	0.52-2.27	0.86	1.14	0.53-2.53	0.74
Timeframe	1996-98	REF[2]			REF[2]		
	2003-05	1.75	0.84-3.67	0.14	1.76	0.83-3.81	0.14
	2010-12	5.90	2.82-12.62	<0.01	5.65	2.60-12.30	<0.01

[1] Numbers for Univariable analysis: Age, sex and timeframe n=252, Ethnicity n=249.

[2]REF is the baseline level (OR = 1.00)

2.5 Discussion

The increase in the proportion of HPV positive cases between 1996-98 and 2010-12 seen in this study augments Ministry of Health data showing over a three-fold increase in OPC case numbers, regardless of HPV status between 1996 and 2010. Overall, the results of this study showed that the majority of OPC cases in New Zealand are caused by HPV. An HPV positive patient is more likely to be under 60. The younger age for HPV positive patients and increase in prevalence from this study are consistent with international data [43,62,89] . Other developed counties have seen increases in the proportion of HPV positive cases ranging from 25% in Sweden between 1970 and 2007 [45], to 225% in the United States between 1988 and 2004 [46]. Moreover, there were no significant differences between the national dataset and study population in terms of patient age, sex, ethnicity, or timeframe of diagnosis, showing this study's results can be extrapolated to the wider New Zealand setting.

There were a higher proportion of deceased patients in our population than the national dataset (59% versus 45%) and this is likely due to the different consenting requirements. Due to the poorer prognosis of HPV negative tumours [50], the inclusion of a higher proportion of deceased cases may have caused an over-representation of HPV negative cases. A small regional New Zealand study performed as a retrospective audit, which did not require any patient consent, showed an overall prevalence of HPV positive OPC of 63% based on p16 results alone [129]. Our overall prevalence of HPV positive cases using an accepted p16/PCR algorithm [89] was 77.9%. It is therefore unlikely that HPV negative cases were overrepresented in our study.

We requested 389 specimens from laboratories throughout New Zealand to capture our final study population of 267 cases. There were several reasons why cases were not retrieved, such as 1) there were logistical difficulties identifying cases (due to the later introduction of computerised records in some centres, and laboratory take-overs/mergers), 2) cases were diagnosed on cytology only and had no biopsy/cell block, 3), specimens had been destroyed in the 2011 Christchurch Earthquake, and 4) specimens had been removed from storage and could not be located. Given the many reasons for samples not being included and the final comparisons of our study population to the national dataset, there was no reason to suspect any systematic bias in our sample.

In New Zealand, laboratories are required by law to report any new diagnosis of cancer to the cancer registry. This generates complete national incidence data. Our study compared only risk factors with data available from the cancer registry (age, sex, ethnicity, and timeframe of diagnosis). It is important to note these are not the only risk factors for HPV positive OPC and other significant factors such as alcohol consumption, tobacco smoking and sexual behaviours [41,61,62] could not be assessed. We therefore cannot rule out possible differences between study participants and those excluded from the study.

It should be noted, that our final criteria for a positive result was both p16 positive and either HPV 16 qPCR positive, or a high risk HPV type confirmed by sequencing. These criteria resulted in 172 results from 267 cases for analysis. This approach excluded focally positive cases and those cases that were p16 negative but HPV 16 positive by PCR. The exclusion of focally positive cases, and use of the p16 positive cut-off at 75%

of tumour cells stained, aligned our definition of a p16 positive result with that used in many other studies [44,47,62,88,89,94].

Although p16 is routinely used alone in the diagnostic setting [93], the use of an algorithm incorporating p16 and another method (PCR or ISH) is preferred in research [47,88,89,97].Whilst some studies only perform PCR testing on p16 positive cases [89], a more common approach is for all samples to undergo PCR testing for HPV DNA [44,47,88]. This approach generates four possible result categories: p16 positive/HPV positive, p16 negative/HPV negative, and the equivocal results of p16 positive/HPV negative and p16 negative/HPV positive [136]. Discrepancies between p16 and PCR results have been reported to be between 6% and 31% [88,99]. In this study, discordant p16/PCR results were seen in 12.8% (25/195) of our cases where, eight cases were p16 positive/high risk HPV negative, and 17 cases were p16 negative/ HPV16 positive. In the p16 positive/HPV negative cases, it is possible that p16 is upregulated by non-HPV related mechanisms [86].

The viral load in tonsillar cancer has been reported to be between 1.54×10^2 and 1.34×10^7 copies per 50 ng of DNA [137]. Our HPV 16 qPCR was capable of detecting 26 copies of HPV, therefore it is unlikely that low levels of HPV are the reason for the eight p16 positive/high risk HPV negative results. Additionally, due to using a new blade for each sample section, and the inclusion of control DNA extractions in each run it is unlikely the p16 negative/HPV positive cases are due to contamination. These 17 cases may represent a transient/bystander HPV infection unrelated to the tumour [47,136]. Junor *et al* [99] reported that p16 negative, HPV DNA positive cases were a distinct clinical entity based on survival characteristics compared to p16 positive/HPV

positive and p16 negative/HPV negative cases. Evans *et al* [88] also reported intermediate survival characteristics for patients with equivocal p16/HPV results. Our criteria for a negative p16 result were less than 10% of tumour cells stained. Interestingly 88% (15/17) of the HPV DNA positive/p16 negative samples showed patchy staining in less than 10% of tumour cells.

While multi-levelled result categories or scoring systems for p16 have been reported [98,101,102], more frequently cases with less than 70% of tumour cells stained are regarded as negative [44,88,89]. Our study had 7.9% (21/267) focally positive p16 cases which had variable staining patterns, and the percentage of cells stained ranged from 10 – 80%. The single case with 80% of cells stained was defined as focally positive as staining was only present in the cytoplasm. This is consistent with two previous studies by Chen *et al* [101] and Lewis *et al* [102] who reported 8.2% and approximately 4%, respectively, of OPC cases with partial p16 staining. The focal staining in both of these studies ranged from less than 5% of tumour cells to 90% of tumour cells, where the cases showing over 75% staining were considered focally positive based on additional criteria such as staining intensity [101,102]. Approximately half of our focally positive cases had HPV DNA detected by PCR. However, further work is required to determine if these cases contain transcriptionally active HPV and therefore are true HPV positive cases, or additional equivocal cases. Regardless, the differing published criteria and lack of international guidelines for p16 interpretation in OPC show clarity is needed. A simple cut off point may not be enough and other factors such as staining intensity, location, and confluence may need to be considered [101,102]. It is possible the age of the blocks contributed to the focally positive results in this study. When looking at each timeframe 5% from 2010-12 were focally positive,

compared to 10% from 2003-05 and 15% from 1996-98. A reduced sensitivity and specificity of p16 on older specimens has been reported by Chenevert *et al* [62], however, their blocks were considerably older and dated back to 1956.

Worldwide HPV 16 is responsible for over 90% of HPV positive OPC [43]. Co-infection is rarely described, and when present usually involves HPV 16 and another high risk type [44,47,138]. To date only HPV 16 is a recognised carcinogen in the oropharynx, while there is limited evidence for HPV 18 [6]. Co-infection was not assessed by this study. However, our findings of HPV 16 accounting for 98.5% of HPV positive cases, and the detection of HPV 33 and 35 is consistent with international data [138]. This study had one case in which the HPV type/s could not be determined. This case was p16 positive, HPV 16 negative and GP5+/6+ positive. The GP5+/6+ PCR is capable of detecting a broad spectrum of HPV types [134]. It is possible the sample contained a low risk HPV type not related to the cancer. The coincidental detection of low/intermediate risk types such as 6, 11, 32, 44, 53, 57 and 81 in OPC has been reported in a systematic review of HPV types in HNSCC by Kreimer *et al*, with HPV 6 the most commonly detected with a prevalence of 3.1% [138]. It is also possible this case contained a co-infection and therefore the types present could not be established by sequencing.

Based on our results, a HPV positive patient is more likely to be under 60, and diagnosed recently (2010-12). The prevalence of HPV positive OPC in females appears to be increasing at a faster rate than in males based on our study data. However, interpretation of this apparent trend is limited due to the low number of cases in females in this study. There were no HPV positive cases in females in 1996-98, although there was one case that was p16 positive that could not have DNA extracted.

This is compared to 20% of p16/HPV DNA positive being in females in the 2010-12 time period. A statistically significant increase in New Zealand OPC cases in females of 2.1% per year between 1982 and 2010 has been previously reported [131]. Although Elwood *et al*, focused on tumour site alone without assessing HPV status, the results support the apparent increase seen in our study.

Chaturvedi *et al* [46] estimated that by 2020 the incidence of HPV positive OPC cases will outnumber the number of cervical cancer cases in the United States. Our data suggests the same scenario will likely be seen in New Zealand. The increasing prevalence of HPV positive OPC and the predominance in males supports the recent inclusion of males in the funded immunisation schedule for Gardasil ®9 in New Zealand. HPV types 16, 18, 6, 11, 31, 33, 45, 52, and 58 are included in Gardasil®9 [109]. Therefore, 99.3% of HPV positive cases with a detectable HPV type in this study contain types included in the vaccine. These results suggest that with adequate coverage, vaccination is likely to have a considerable impact on the future incidence of HPV positive OPC in New Zealand

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate:

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Name of Published Research Output and full reference:

In which Chapter is the Published Work:

Please indicate either:

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  and / or

- Describe the contribution that the candidate has made to the Published Work:

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Candidate's Signature

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Date: 2018.07.05 10:21:12 +12'00'

Principal Supervisor's signature

Date

Chapter 3 - Evaluation of p16 and CK19 immunohistochemistry, nucleic acid detection and viral load in archived formalin fixed paraffin embedded biopsy samples for the detection of HPV in oropharyngeal cancer

3.1 Abstract

Background and aims: HPV causality in oropharyngeal cancer is often determined using p16 immunohistochemistry alone. However, discrepancies between p16 and HPV status by PCR occur in up to 30% of cases. As a result, the HPV status of these discrepant cases is often uncertain. Thus, the aim of this research was to compare HPV detection using a range of biomarkers in oropharyngeal cancer biopsies with variable p16 results.

Methods: This study used archived oropharyngeal cancer biopsies from a previous study. Cases comprised 24 p16 positive HPV 16 DNA positive, 24 p16 negative HPV 16 DNA negative, ten p16 negative HPV 16 DNA positive, and 18 focally positive p16 samples of which nine were HPV 16 DNA positive. CK19 immunohistochemistry was performed on all specimens with adequate tumour sample (n=68) and H-score calculated (Percentage cells stained x staining intensity). RNA extraction, RT-PCR for human beta-globin and HPV 16 E7, and viral load per copy of human beta-globin was performed on all DNA positive samples (n=43). For p16 focally positive samples the percentage, intensity, location and confluence of staining was recorded.

Results: Amplifiable beta-globin mRNA was detected in 28/43 HPV 16 DNA positive samples. Of these 16 were HPV 16 E7 mRNA positive. Of the 16 E7 positive specimens 12 were p16 positive, two were p16 focally positive and two p16 negative. A cut off value of an H-score of 60 was used to determine a CK19 positive result. There were 33 negative and 35 positive CK19 results. Viral loads could be calculated for 33/43 HPV 16 DNA positive samples and ranged from 0.01 to 285.63 copies of HPV 16 E7 per copy of human beta globin, with a median viral load of 1.69 copies. There was moderate

agreement between p16 and CK19 (κ =0.45, 95%CI: 0.23-0.66), and CK19 and HPV 16 DNA (κ =0.47, 95% CI: 0.23-0.71). Staining in the p16 focally positive specimens was variable in percentage of tumour stained, intensity and location of staining. All focally positive samples showed confluent staining in groups of greater than ten cells. A total of ten cases would have had the tumour HPV status incorrectly classified if p16 alone was used.

Conclusions: These results suggest using p16 alone to determine HPV status in OPC has limitations which require serious consideration before de-escalated therapies for HPV positive cases are applied.

3.2 Introduction

The incidence of head and neck cancer is decreasing in many developed countries in parallel with the decrease in the number of individuals who smoke [40,41]. However, in these same countries, the incidence of oropharyngeal cancer (OPC) is increasing [42,43]. Worldwide, it is estimated that 26% of oropharyngeal cancers are caused by HPV [43]. However, the percentage of OPC caused by HPV is much higher in some countries. For example, in Sweden 93% of cases were HPV positive in 2006-07 [45] and in New Zealand 88% of cases were HPV positive in 2010-12 [139]. HPV positive OPC tend to be seen in younger patients who are non-smokers and light drinkers, with sexual behaviours consistently reported as risk factors [41,43].

HPV positive OPC show increased responsiveness to chemo- and radio- therapy than HPV negative cases [49]. In addition, disease free survival and overall survival is

significantly improved in HPV positive patients [50]. The improved prognosis of HPV positive OPC has led to investigations around the de-intensification of treatments [53]. Current treatment regimens such as intensified radiation and concurrent chemo-radiotherapy can result in significant toxicities and long term morbidities including: severe dysphagia, dependence on a feeding tube, facial disfigurement, speech difficulties and changes in salivary output, pH and viscosity [54-56]. The goal of de-intensification is to maintain current cure rates while minimizing long term morbidities [53]. Many de-intensification studies have determined HPV status using p16 alone [53]. However, the use of p16 alone is problematic as differences in the survival of patients with p16 positive tumours, with and without HPV E6/E7 positive serology have been reported [100]. The accurate determination of HPV status is essential before these personalised treatment approaches can be applied.

The gold standard for HPV detection is the detection of E6/E7 mRNA [86]. Detection of mRNA confirms not only the presence of the virus but that it is transcriptionally active and therefore considered causative [47,86]. E6/E7 mRNA detection is costly and time consuming and is not well suited to the usual formalin fixed paraffin embedded (FFPE) specimens seen in the diagnostic laboratory [86].

In the diagnostic setting, HPV positivity in OPC is often reported based on p16 testing alone [93]. p16 is a tumour suppressor protein upregulated due to disruption of the retinoblastoma pathway by the E7 protein of high risk HPVs [93]. It is important to note there is no direct link between HPV and p16, and as such p16 is a surrogate marker of HPV infection [86]. It is possible p16 is over-expressed due to non-HPV

mechanisms [86], and furthermore p16 negative cases have been shown to harbour transcriptionally active HPV [107].

Further complicating the use of p16 is the lack of clear guidelines on what constitutes a positive result. Many authors use a cut-off point of 70% of tumour cells showing strong and diffuse staining for a positive result [44,47,62,88,89]. It has been described that if a cut-off of 75% of tumour cells showing strong and diffuse staining is used the samples will always contain HPV mRNA [94]. Others argue a simple cut-off point is not enough and staining intensity and confluence should be considered [101,102]. While the majority of cases are clearly p16 positive or negative, a small proportion of cases show focally positive staining, and the causative role of HPV in these cases is uncertain.

These focally positive cases highlight the need for the development of additional markers for tumour HPV status. It has been suggested that cytokeratins may be useful markers of HPV status [140,141]. The actions of high risk HPV E7 proteins result in disruption of cytokeratin expression, in particular cytokeratin 19 (CK19) due to the immortalization of keratinocytes [141]. Another possible marker may be the viral load of the tumour. To date viral load has been used in several studies as a prognostic marker for HPV positive OPC [78,107,142].

Our previous work (Chapter 2) identified cases with focally positive p16 staining and discrepancies between p16 and HPV DNA results [139]. Thus, the aim of this research was to compare HPV detection using a range of biomarkers on historic oropharyngeal cancer biopsies with variable p16 results.

3.3 Materials and methods

Ethics approval was obtained from the Health and Disabilities Ethics Committee (reference: 14/STH/128). Participant privacy was upheld by de-identification of samples.

3.3.1 Participant recruitment and sample collection

A flow chart of cases used and specimen testing is shown in Figure 3.1. Cases used in this study were enrolled previously (Chapter 2) [139]. Briefly, cases were identified by a New Zealand cancer registry search of all cases of oropharyngeal cancer of ICD-10 codes C01, C05.1-5.2, C09.0-9.9, and C10.0-10.9 from 1996-98, 2003-05, and 2010-12. Informed written consent was provided from participants still living. In addition the whānau (extended family) of deceased participants identified as Māori from the Auckland region were requested to give consent for these specimens to be used, as per local Iwi (tribe) wishes. After specimen requests were made, laboratories sent the specimens and a copy of the pathology report to Massey University, Palmerston North, New Zealand.

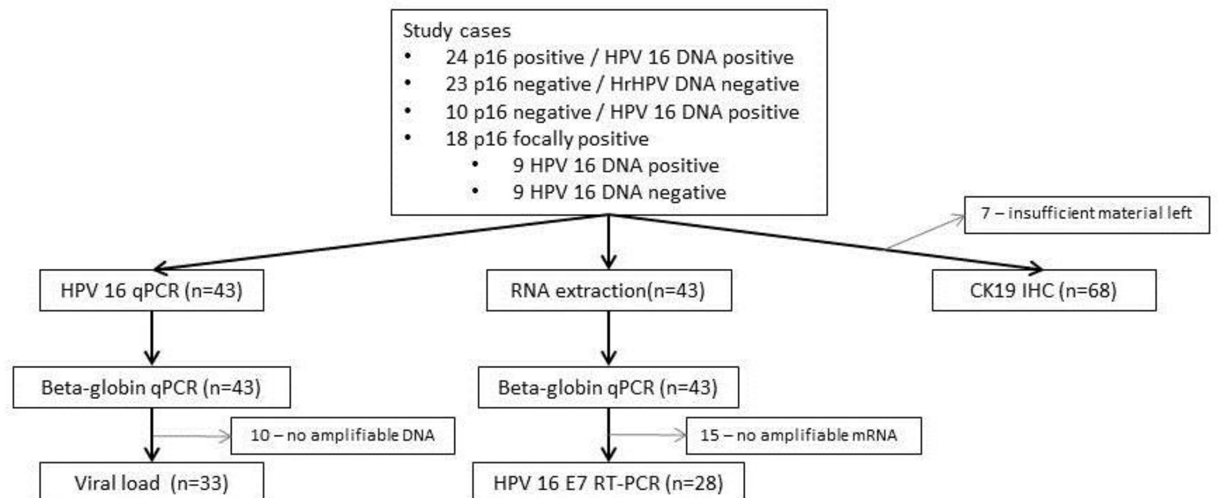

Figure 3.1: Flowchart of study cases and testing performed.

3.3.2 DNA extraction

A 10 µm slice of representative FFPE tumour block was cut for DNA extraction using a new blade between each sample to avoid cross contamination of samples. DNA extractions were performed using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) as per manufacturer's instructions for paraffin embedded tissue. A tissue-free extraction using all kit reagents was included with each set of samples. The tissue-free extractions were then used as negative controls in the beta globin and HPV 16 qPCRs. All samples were eluted in a final volume of 100 µL. The quality of extracted DNA was assessed using a Nanodrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

3.3.4 RNA extraction

A 10 µm slice of representative FFPE tumour block from each HPV 16 DNA positive case was cut for DNA extraction using a new blade between each sample to avoid cross

contamination of samples. RNA extractions were performed using the RNeasy® FFPE Kit (Qiagen) as per manufacturer's instructions, with a deparaffinisation step using xylene. A final elution volume of 25 µL was used. A tissue-free extraction was included with each set of samples. The tissue-free extractions were then used as negative controls in the beta globin and HPV 16 qPCRs. The quality and quantity of extracted RNA was assessed using a Nanodrop™ Spectrophotometer (Thermo Fisher Scientific). Extracted RNA was stored at -80 °C until processing.

3.3.5 Beta-globin qPCR

The presence of amplifiable nucleic acid (DNA and RNA) was assessed on all cases by qPCR targeting the human beta-globin gene using the PC03 and PC04 primers [133] as previously described [139]. A sample was considered positive if it produced a T_m of 81.0°C (±1.0°C) after 40 cycles.

3.3.6 HPV 16 DNA qPCR and viral load calculations

The presence of HPV 16 DNA was assessed as previously described [139]. A sample was considered positive if it crossed the threshold and produced a T_m of 77.6°C (±1.0°C). Viral load on HPV 16 DNA positive samples was calculated per copy of the human beta-globin gene. The HPV 16 positive control was a previously described cloned plasmid that contained an approximately 1.8 kb fragment of the HPV 16 genome that included the E6 and E7 gene sequence [139]. The beta-globin control was a previously described plasmid that contained an approximately 665 bp fragment of the human beta-globin gene sequence amplified using the GH20 and GH21 primers [133]. Copy numbers for HPV 16 and beta-globin were determined in duplicate by using standard curves, generated in the same PCR run with either the HPV 16 or beta-globin plasmid.

Standard curves were made from six 10-fold serial dilutions with the beta-globin curve starting at 2.03 x 10^6 copies, and the HPV 16 curve starting at 1.77 x 10^5 copies. Serial dilutions of a beta-globin clone control identified that the assay was sensitive down to 13 copies of the target sequence. Serial dilutions of the HPV 16 clone control identified that the HPV 16 DNA assay was capable of detecting as few as four copies of the HPV 16 E7 target sequence. Viral load was calculated by dividing the mean HPV 16 copy number by the mean beta globin copy number. HPV DNA viral load was defined as the number of HPV DNA copies per beta-globin gene copy.

3.3.7 RT-PCR

Before cDNA synthesis extracted RNA was subject to the HPV 16 E7 qPCR, as described below, to check for contaminating DNA. If DNA was present an additional DNase treatment was performed. cDNA was synthesized using the First Strand Transcriptase cDNA synthesis kit (Roche). Reactions were as per manufacturer's instructions. cDNA was used in the beta-globin and HPV 16 E7 qPCR's.

3.3.8 HPV 16 E7 qPCR

The presence of HPV 16 E7 was assessed on all cases with a positive HPV 16 DNA result. A qPCR with melt curve analysis (protocol shown in appendix 7) was developed using previously described primers and cycling conditions [143]. Each reaction mix contained: 0.15 µM each primer and 1X Fast Start SYBR Green Master (Roche, Basel, Switzerland). PCR was followed by a melt curve from 70 – 80°C with 0.2°C increments and a five second hold. A sample was considered positive if it crossed the threshold and produced a T_m of 78.5°C (±1.0°C). Serial dilutions of the HPV 16 clone control

showed the assay was capable of detecting as few as nine copies of the HPV 16 E7 target sequence.

3.3.9 p16 immunohistochemistry

p16 IHC was performed at a diagnostic medical laboratory (MedLab Central, Palmerston North, New Zealand). FFPE tissue was used for IHC. Sections were cut at four microns and baked onto Superfrost Plus slides at 60°C for 60 minutes. Staining was performed on the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, USA), with antigen retrieval performed on board, using Ventana CC1 buffer for 32 minutes at 100°C. A 1:150 dilution of the p16 (INK4a) antibody (G175-405, product code 551153) (BD Biosciences, North Ryde, Australia), was incubated on the slide for eight minutes at 36°C. The detection of p16 was then visualised using the Ventana Optiview DAB kit (Ventana Medical Systems). A multi-tumour block positive control containing a serous ovarian carcinoma was included with each run of samples. All slides were read independently by two pathologists (BL and UV), and discordant cases reviewed to reach a consensus. Samples showing strong diffuse staining in the nucleus and cytoplasm of greater than 75% of tumour cells were considered positive. Samples showing staining in 10% to 75% of tumour cells were considered focally positive, and samples with staining in less than 10% of tumour cells were considered negative. Samples considered focally positive had the percentage of cells stained, staining intensity, location, and confluence recorded.

3.3.10 CK19 immunohistochemistry

CK19 IHC was performed at a diagnostic medical laboratory (MedLab Central, Palmerston North, New Zealand), FFPE tissue. Sections were cut at four microns and

baked onto Thermo Superfrost Plus slides at 60°C for 60 minutes. Immunostaining was performed on the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, USA), with antigen retrieval performed on board, using Ventana CC1 buffer for 32 minutes at 100°C. A 1:100 dilution of the CK19 (RCK108) antibody (DAKO, product code M0888) (Agilent, Santa Clara, CA 95051 United States), was incubated on the slide for 32minutes at 36°C. The detection of CK19 was then visualised using the Ventana Optiview DAB kit (Ventana Medical Systems). Normal colon was used as a positive control on each slide, and normal kidney used as a negative control. The CK19 H-score was calculated by multiplying the percentage of tumour cells stained by the staining intensity (1+, 2+, or 3+) to give a final score of 0-300. All slides were read independently by two pathologists (BL and CT), and discordant cases reviewed to reach a consensus.

3.3.11 HPV 16 E7 immunohistochemistry

The use of an immunohistochemical assay for HPV 16 E7 detection was also evaluated. Antigen retrieval techniques using CC1 buffer, CC2 buffer, protease, and a combination of heat and protease as previously described [144] were applied. Dilutions of antibody from 1:50 to 1:1600 of the HPV 16 E7 antibodies: clone 8C9 (Thermo Fisher Scientific), TVG701Y (Thermo Fisher Scientific), and ED17 (Santa Cruz Biotechnology) were used. The detection of HPV 16 E7 was then visualised using the Ventana Optiview DAB kit (Ventana Medical Systems). Antibody trials used known HPV 16 positive CIN3, and oropharyngeal cancer biopsies. A multi-tumour block control was included in all trials.

3.3.12 Statistical analysis

Initial exploration of data was by summary statistics, tables and plots. Associations between test results were explored using two by two tables, and plots. Agreement between tests measured using the Kappa statistic, and the potential for systematic bias assessed using the McNemar test [145]. Viral load was normalised using the natural log transformation. Attempts were made to normalise the distributions of CK19 H-scores using log, inverse, square root and log10 transformations. However, all transformations were unsuccessful thus non-parametric statistics were used to investigate the alternative hypothesis that HPV 16 DNA/RNA positive samples would have greater CK19 scores than HPV 16 DNA/RNA negative samples. Descriptive analysis was performed using the software package R version 3.2.0 (R Development Core team, 2010, R Foundation for Statistical Computing, Vienna, Austria).

3.4 Results

The cases used in this study were identified from a previous study (Chapter 2) [139] and comprised 24 p16 positive/HPV 16 DNA positive specimens, 23 p16 negative/HPV 16 negative specimens, ten p16 negative/HPV 16 DNA positive specimens, and 18 focally positive p16 specimens Of the p16 focally positive specimens, nine were positive for HPV 16 DNA, and nine were HPV DNA negative.

3.4.1 Focally positive p16 specimens

Staining in the p16 focally positive specimens was variable (Figure 3.2) with the percentage of tumour cells stained ranging from 10% to 80%. A description of the focally positive specimens is shown in Table 3.1.

3.4.2 HPV 16 DNA and viral load

There were 43 HPV 16 DNA positive specimens in this study. Amplifiable beta-globin was present in 77% (33/43) specimens. The calculated viral load in these specimens ranged from 0.01 to 285.63 copies of HPV 16 E7 per copy of human beta-globin, with a median viral load of 1.69 copies. The remaining ten specimens were omitted from viral load analysis due to a lack of amplifiable beta-globin after long term freezer storage.

3.4.3 HPV 16 RNA detection

A total of 43 specimens were able to have RNA extracted. Twenty-eight specimens had amplifiable beta-globin mRNA. Of these, 16 were positive for HPV 16 E7 mRNA and 12 were negative. Of the 16 specimens positive for HPV 16 E7 mRNA, 12 were p16 positive, two were p16 focally positive and two p16 negative. Fifteen specimens did not have amplifiable beta-globin mRNA, however, six of these specimens were found to be positive for HPV 16 E7 mRNA.

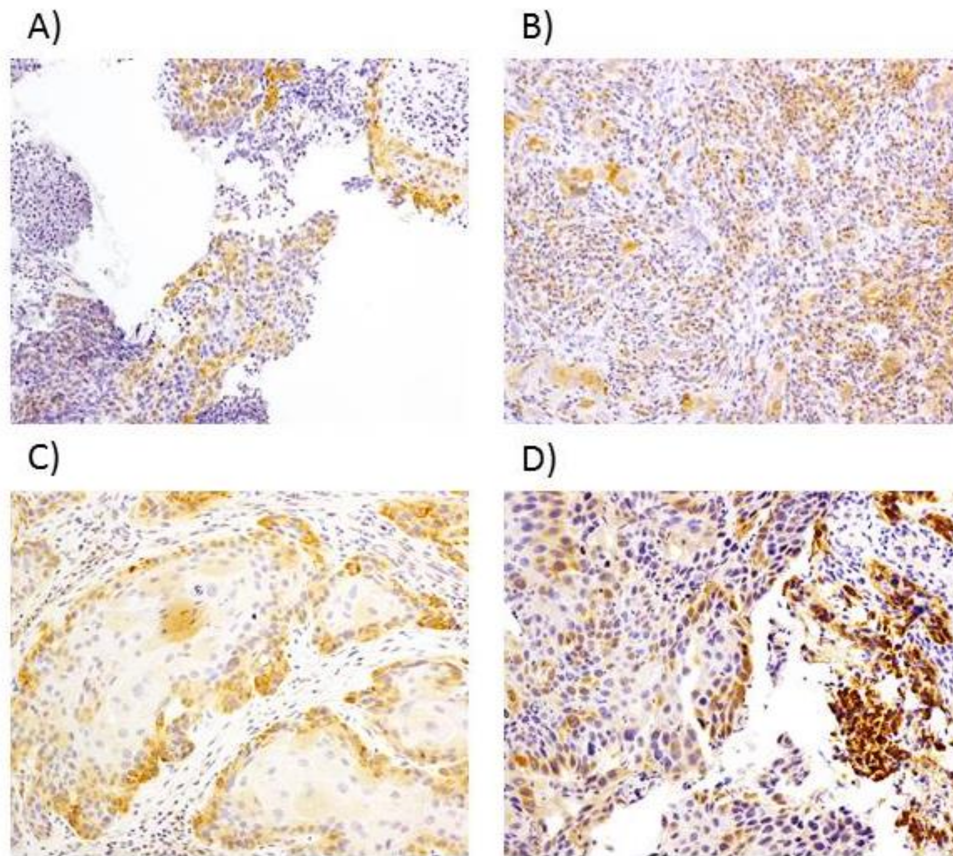

Figure 3.2: Staining patterns of four specimens with focally positive p16 results. Magnification 20x. A) Specimen 7, HPV 16 E7 mRNA positive. B) Specimen 51, HPV 16 E7 mRNA positive but result excluded due to lack of amplifiable beta-globin. C) Specimen 54, HPV 16 E7 mRNA negative. D) Specimen 220, HPV 16 E7 mRNA positive.

Table 3.1: Staining characteristics and HPV 16 DNA, RNA, Viral load, and CK19 results for p16 focally positive specimens.

Case	Percentage of tumour cells stained	Staining intensity	Staining location	Staining confluence	HPV 16 DNA	Viral load[1]	HPV 16 RNA[2]	CK 19 H-Score[3]
220	30	3+	Cytoplasmic	>10 cells	Positive	23.23	Positive	-
7	20	1+	Cytoplasmic	>10 cells	Positive	1.69	Positive	300
54	30	2+	Cytoplasmic	>10 cells	Positive	285.63	Negative	0
59	80	2+	Cytoplasmic	>10 cells	Positive	0.03	Excluded	60
25	50	3+	Cytoplasmic	>10 cells	Positive	0.02	Excluded	80
30	20	2+	Cytoplasmic	>10 cells	Positive	0.34	Excluded	0
10	10	2+	Cytoplasmic	>10 cells	Positive	0.01	Excluded	0
47	50	2+	Cytoplasmic	>10 cells	Positive	-	Excluded	0
51	80	2+	Cytoplasmic	>10 cells	Positive	-	Excluded	80
21	20	2+	Cytoplasmic	>10 cells	Negative	-	-	0
23	40	3+	Nuclear + Cytoplasmic	>10 cells	Negative	-	-	80
49	25	3+	Cytoplasmic	>10 cells	Negative	-	-	120
52	15	3+	Nuclear + Cytoplasmic	>10 cells	Negative	-	-	0
146	25	2+	Cytoplasmic	>10 cells	Negative	-	-	-

Case	Percentage of tumour cells stained	Staining intensity	Staining location	Staining confluence	HPV 16 DNA	Viral load[1]	HPV 16 RNA[2]	CK 19 H-Score[3]
125	50	2+	Nuclear + Cytoplasmic	>10 cells	Negative	-	-	100
238	40	1+	Cytoplasmic	>10 cells	Negative	-	-	-
243	30	3+	Cytoplasmic	>10 cells	Negative	-	-	40
157	40	2+	Nuclear + Cytoplasmic	>10 cells	Negative	-	-	0

[1] Missing values due to a lack of amplifiable beta-globin after long term storage.

[2] Specimens were excluded due to a lack of amplifiable beta-globin, regardless of HPV 16 E7 mRNA result.

[3] Missing values due to specimens having no remaining tumour left in the block for testing.

3.4.4 CK19 immunohistochemistry

Sixty-eight specimens were evaluated for the expression of cytokeratin using the marker CK19. CK 19 H-scores ranged from zero (no staining) to 300 (strong staining in all tumour cells), as shown in Figure 3.3 with a mean score of 105.3, a median of 60 and lower and upper quartiles of 0 and 202.5 respectively. A cut-off of 60 was used to define positive and negative. Using this cut-off there were 33 negative, and 35 positive CK19 results.

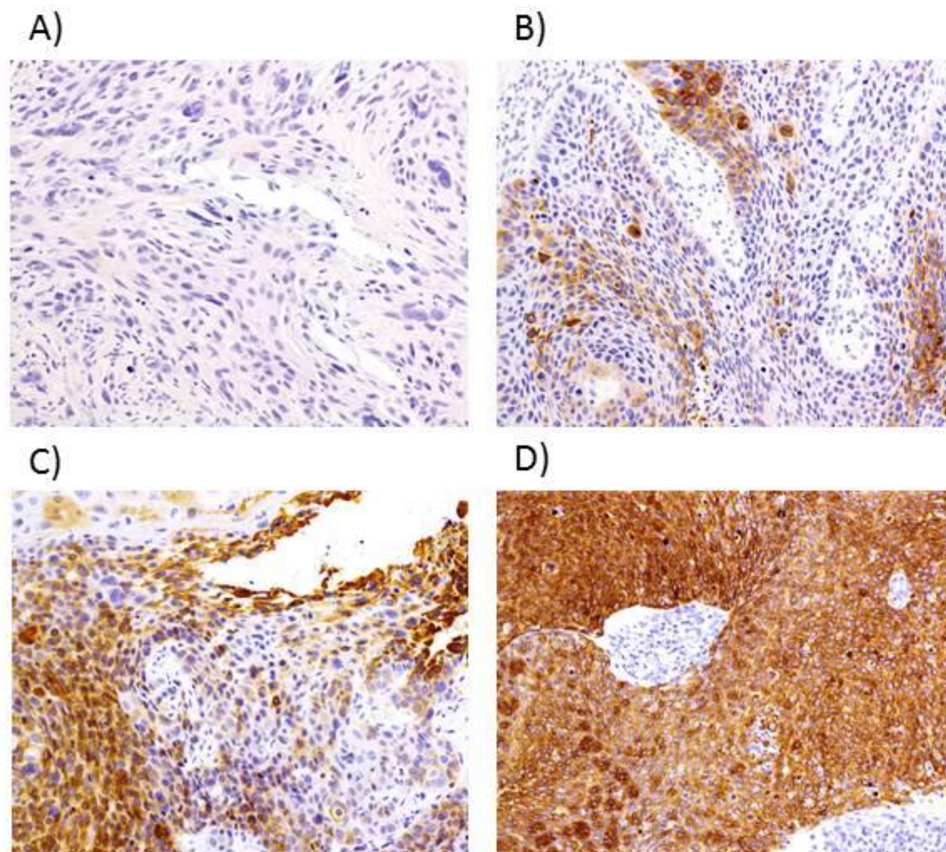

Figure 3.3: CK19 immunohistochemistry image panel. Magnification 20x. A) CK19 H-score of 0/no staining in tumour cells. B) & C) Staining of varying intensity in some tumour cells. H-scores of 60 and 100. D) CK19 H-score of 300/3+ intensity staining in all tumour cells.

3.4.5 HPV 16 E7 immunohistochemistry

Unfortunately, the HPV 16 E7 assay was not able to be optimised due to non-specific staining seen throughout the multi-tumour block with all tested antibodies. There appeared to be some specific staining with the TVG701Y antibody, however this was substantially reduced when the assay was altered to attempt to reduce the non-specific staining.

3.4.6 Test comparisons

The sensitivities and specificities of p16 and CK19 compared to the gold standard of HPV 16 E7 mRNA detection are shown in Table 3.2. p16 appeared to perform better than CK19, with similar sensitivities (75% versus 77%), but a higher specificity (50% versus 27%) although this difference was non-significant as shown by the broad confidence intervals. There was no advantage seen using a combined p16/CK19 algorithm.

Table 3.2: Sensitivities and specificities of p16 and CK19 compared to the gold standard of E7 mRNA detection.

Test	Sensitivity (95% CI)	Specificity (95% CI)
p16	75% (48% - 93%)	50% (21% - 79%)
CK19	77% (46% - 95%)	27% (6% - 61%)
p16 and CK19	80% (44% - 97%)	38% (9% - 76%)

The agreement between tests was assessed using the kappa statistic (Table 3.3). There was moderate agreement between p16 and CK19 (0.45, 95%CI: 0.23-0.66), and CK19 and HPV 16 DNA (0.47, 95% CI: 0.23-0.71). A statistically significant McNemar test statistic was found when comparing CK19 and p16 (p<0.01) indicating there was potential for a systemic difference between the proportion of positive results between the two tests. The McNemar test statistic was not significant for all other test comparisons.

The relationship between the log of viral load and p16 status, CK19 result and HPV 16 RNA detection is shown in Figure 3.4.

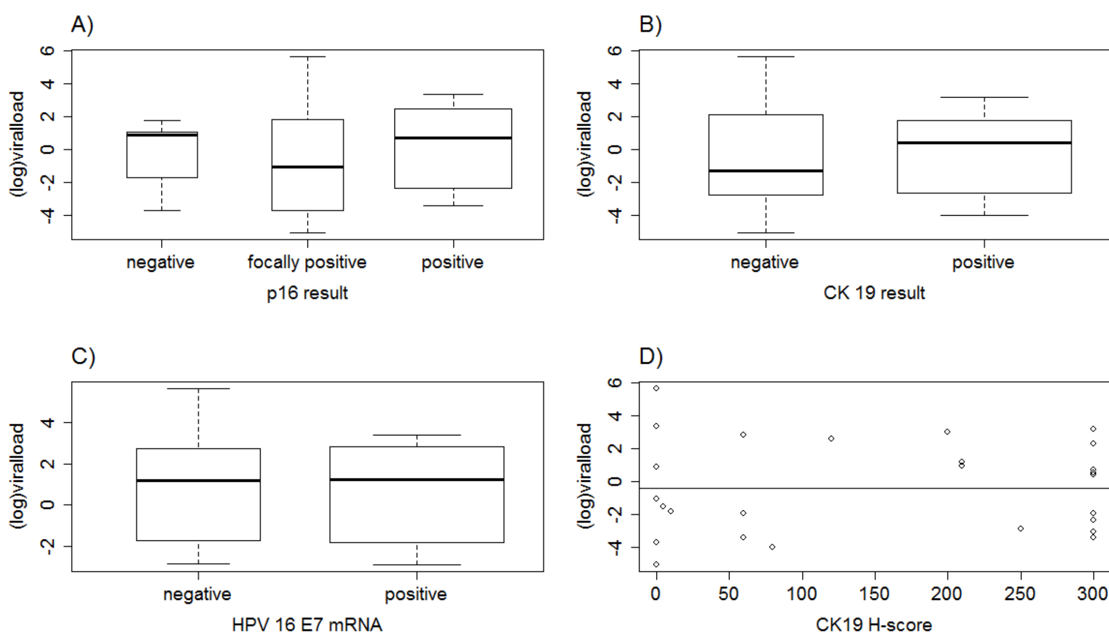

Figure 3.4: Viral load boxplots and scatterplot. A) (log) Viral load and p16 result. B) (log) Viral load and CK19. C)(log) Viral load and HPV 16 E7 mRNA. D) scatterplot of (log) Viral load and CK 19 H-score with regression line.

There was no association between the log of the viral load and CK 19 H-Score. Viral load appeared to be higher in CK19 positive samples when the cut off was applied (Figure 3.3 B), however, this was not statistically significant.

When comparing CK19 and DNA and RNA detection there was no difference (p = 0.42) between the CK19 H-scores of HPV 16 E7 RNA positive and negative cases (Table 3.4). However, a difference was observed between the H-scores of HPV DNA positive and negative cases with a Wilcoxon rank sum test p value of <0.01

Table 3.3: Kappa statistics, and 95% confidence intervals showing agreement between tests.

Tests compared	Kappa statistic	95% confidence interval	Agreement
p16 / CK19	0.45	0.23 – 0.66	Moderate
p16 / HPV 16 E7 mRNA	0.26	0 – 0.62	Fair
CK19 / HPV 16 E7 mRNA	0.04	0 – 0.40	Slight
CK19 / HPV 16 DNA	0.47	0.23 – 0.71	Moderate

Table 3.4: Comparison of CK19 H-scores and HPV 16 E7 mRNA and DNA detection.

		n	Min.	1st Qu.	Median	3rd Qu.	Max.	Mean (95% CI)
HPV 16 E7 mRNA	Positive	13	0.0	60.0	200.0	300.0	300.0	167.7 (92.5-242.9)
	Negative	11	0.0	32.5	200.0	270.0	300.0	165.0 (81.1-248.9)
HPV16 DNA	Positive	37	0.0	10.0	200.0	300.0	300.0	157.4 (116.2-198.6)
	Negative	31	0.0	0.0	0.0	65.0	300.0	43.1 (15.4-70.7)

3.5 Discussion

The description of cases with focally positive p16 staining in previous work (Chapter 2) led to this study, as the causative role of HPV in these cases was uncertain. Although the role of HPV in a number of these cases is still uncertain due to problems with RNA tests, we have identified four cases that, based on p16 alone, would have been called HPV negative, although they contain transcriptionally active HPV. It has previously been suggested that p16 staining patterns correlate with HPV status [101,102]. In particular, diffuse nuclear and cytoplasmic staining, regardless of intensity, correlated with the presence of HPV DNA [101], or >50% of tumours cells stained with >25% confluent areas was correlated with the presence of HPV mRNA [102]. Of the two focally positive p16 specimens that contained transcriptionally active HPV 16 in our study, one had 30% of tumour cells stained with 3+ intensity, while the other had 20% of tumour cells stained with 1+ intensity. The probable HPV positive case, with a negative beta-globin result, had 80% of cells stained with 2+ intensity. All three of these specimens showed staining in the cytoplasm of tumour cells only. By comparison, none of the four focally positive specimens with nuclear and cytoplasmic staining contained HPV DNA. These results highlight the complexities of using a surrogate marker and that a simple arbitrary cut-off does not provide accurate HPV status in all cases.

RNA isolated from FFPE blocks is often of low quality due to RNA degradation prior to fixation, and the cross-linking between proteins and nucleic acids caused by formalin fixation [146]. In this study, 35% (15/43) of cases lacked amplifiable nucleic acid after RNA extraction and RT-PCR. However, of these samples, six of the 15 were positive for HPV 16 E7 mRNA. This difference may be due the length of the amplicon in each PCR.

The beta-globin amplicon was 110bp [133], compared to the 75bp amplicon for the HPV 16 assay [143]. The smaller size of the HPV 16 amplicon may have allowed for better detection of heavily fragmented target sequences.

Cytokeratins are proteins found in the cytoskeleton of epithelial tissues and their expression is dependent on tissue type and maturity of the epithelium [141]. CK19 is the lowest weight cytokeratin and is expressed in the basal layer of mucosal epithelium [141]. CK19 is known to be expressed in high grade cervical pre-cancerous lesions, invasive cervical carcinoma [140], and has been shown to be more highly expressed in HPV positive compared to negative oropharyngeal cancers. Therefore, CK19 may be a useful marker for the determination of tumour HPV status [141]. This study used a cut-off of a CK19 H-score of 60 to define a positive result. In a study of 38 oropharyngeal and oral cavity cancers, Santoro *et al* found all cases with integrated high risk HPV had H-scores of 270-300 [141]. Our study found there was no significant difference between the H-scores in RNA positive and negative tumours with a p-value from the Wilcoxon rank sum test of 0.42. The only difference was observed when comparing H-scores to HPV 16 DNA detection ($p < 0.01$). The comparison of CK19 H-score to the presence of integrated virus as undertaken by Santoro *et al* may be problematic as it is reported that in over half of OPC cases HPV is present in the episomal form only [78,79,147]. Therefore, comparisons of CK19 expression and viral integration are likely to not accurately describe the relationship between CK19 and tumour HPV status as the physical form of the virus is not a determining factor in the development of OPC. It appears there is some association between CK19 and HPV infection [140,141], but its role as a marker of tumour HPV status is uncertain and requires further investigation with larger studies.

Viral load has been used in several studies to examine the prognosis of HPV positive OPC [78,107,142]. It has been found that tumours with a higher viral load show an improved prognosis compared to low viral load tumours [78,107]. Furthermore, HPV viral load in advanced OPC has been shown to be significantly associated with response to induction chemotherapy, concurrent chemo-radiotherapy, overall survival and disease free survival [142]. Although viral load may be useful as a prognostic marker, we found no differences between the viral load in RNA positive and negative cases, suggesting the role of viral load as a diagnostic marker for OPC is limited.

At the current time, the misclassification of the HPV status of an oropharyngeal tumour is not likely to have any significant affects for the patient. However, de-escalation of therapies for HPV positive tumours is currently being investigated in clinical trials [53]. This study identified four patients who would have currently been misclassified as HPV negative, two with focally positive and two with negative p16 results. In addition, a further six cases would have been falsely identified as HPV positive based on a positive p16 result. These results are consistent with previous studies. For example, Holzinger *et al* found transcriptionally active HPV 16 in 22% of p16 negative OPC, and no transcriptionally active HPV in 21% of p16 positive cases [107]. If de-intensified therapies were in fact being used, this would mean four patients could have had unnecessarily high intensity treatment and therefore a higher risk of long term morbidities [56], and a further six may have wrongly received de-intensified treatments which may have had an impact on their cure rate [53,56].

There is controversy over whether it is HPV or p16 status that confers the better prognosis for OPC patients. Lewis *et al* argue p16 status regardless of HPV status is the

key to identifying the largest number of patients with favourable prognoses [148]. It was shown by Ang *et al* that p16 expression with a cut-off of 70% of tumour cells showing strong and diffuse nuclear and cytoplasmic staining was a stronger prognostic factor for both overall survival and progression free survival, than HPV detection by ISH [52].Two studies have shown cases with discrepant p16/HPV results to have intermediate prognoses between p16/HPV positive and p16/HPV negative cases [88,99]. However, a recent meta-analysis of 18 controlled clinical trials comparing p16 positive and negative oropharyngeal cancers found four studies that excluded cases with discrepant p16/HPV status from analysis [149]. Although there are only a small number of these discrepant cases, it is important we can accurately determine the HPV status in all cases if personalised therapies are to be used in the future.

The ideal test for the HPV status of a tumour should be able to detect transcriptionally active HPV, and also be suited to laboratory workflow [86]. The surrogate marker, p16 is low cost, accessible and a useful marker for HPV [86,93]. However, the lack of any direct link between p16 overexpression and high risk HPV is problematic. The detection of HPV E6 or E7 proteins by immunohistochemistry would in theory provide a highly sensitive and specific test. Although this has been described [144,150], we were unsuccessful in the optimisation of an HPV 16 E7 immunohistochemical assay in this study. The advantage of an immunohistochemical test is that it aligns with the specimen types and equipment readily available in many histopathology laboratories. The current gold standard of E6/E7 mRNA detection is best suited to fresh tissue, and requires molecular equipment, reagents and expertise not present in all laboratories [86]. In a country such as New Zealand with a low population density, many histopathology departments are small and part of hospital laboratories offering only

core services. Any molecular based tests are therefore performed at reference laboratories. This impacts on the practicalities of fresh tissue and further highlights the need for the development of additional tests for determining HPV status in oropharyngeal cancer.

Chapter 4 - Descriptive analysis of a pre-vaccine population of oropharyngeal cancer patients in New Zealand

4.1 Abstract

Background and Aims: HPV positive oropharyngeal cancer is consistently linked to sexual behaviours whereas HPV- negative cases tend to be associated with tobacco and alcohol consumption. There is currently no description of risk factors for HPV positive oropharyngeal cancers in the New Zealand population. This study aimed to describe the demographics and exposures of a pre-vaccine population of oropharyngeal cancer patients and determine risk factors for HPV positive oropharyngeal cancer.

Methods: Participants were recruited prospectively through Ear Nose and Throat surgeons (n=45) and retrospectively through a previous study (n=44). p16 results were provided in diagnostic pathology reports and from previous research. Each participant completed a questionnaire covering demographics, substance exposures, lifestyle factors, sexual behaviours, and medical history. Associations between putative risk factors and p16 positive OPC were investigated using logistic regression, and a multivariable model built retaining factors significant at $p<0.05$. Free text comments were analysed using inductive content analysis to derive themes.

Results: The study population consisted of 89 patients who completed questionnaires; 79 had cancers that were p16 positive, eight were p16 negative, and two did not have reported p16 results. Compared to the New Zealand working survey, study participants showed significantly higher exposures to asbestos and formalin. There was a significant association between a p16 positive result and having ever had oral sex (Adjusted OR: 15.9, 95% CI: 1.4-230.5). Analysis of the 32 free text comments generated two

themes: participants identified factors that contributed to their cancer (n=16), and the importance of personal recognition of symptoms (n= 11).

Conclusions: The association between oral sex and HPV positivity is consistent with international data. The role of occupational exposures in oropharyngeal cancer, and possible interactions between substances and HPV, that may alter the course of infection warrants further research.

4.2 Introduction

Head and neck cancer was traditionally associated with tobacco and alcohol use and seen in older patients [43]. The incidence of head and neck cancers as a group has decreased in parallel with the decrease in the number of individuals who smoke [40,41]. Over the last few decades, and increasing number of cancers of the oropharynx (including tonsils and base of tongue) have been seen in younger patients who are non-smokers and light drinkers [41]. In some populations, as many as 90% of oropharyngeal cancers (OPC) are caused by human papillomavirus (HPV) [45,139]. HPV is a common sexually transmitted virus and the majority of sexually active adults have been infected with HPV at some point in their life, with the majority of infections being cleared by the immune system [14]. In a small proportion of infected individuals, persistent infection with a high risk HPV type can lead to the development of cancer [9].

The most studied risk factor for HPV positive OPC is sexual behaviour, and it is thought the increasing incidence of HPV positive OPC is due to changing sexual behaviours [42,61]. These changes have occurred over generations as a result of the increased

availability of contraception, sexual expression in the media, and rebellion of the younger generation [62]. This idea is supported by data from the US, Australia, and Sweden which have shown an increase in the occurrence of premarital sex, oral sex, the number of lifetime partners, and a reduction in the age of sexual debut in recent birth cohorts [49].

Tobacco use and alcohol have strong associations with HPV negative head and neck cancer but their associations with HPV positive cases are inconsistent [50]. When limiting the association to only oropharyngeal cancer, as opposed to all head and neck cancers, no associations between alcohol and or tobacco have been found [61,63,67,68]. Other risk factors for HPV positive OPC have been described and include marijuana use [63], diet and nutrition [72].

Associations between occupational exposures to hazardous substances and head and neck cancer have been described [151-154]. The most described is asbestos exposure with an OR of 1.9 (95% CI: 1.6-2.3) for head and neck cancer in those exposed to asbestos [153]. However, the studies of occupational exposures and head and neck cancer to date have not explored any associations between occupational exposures and the HPV status of tumours.

Additionally, there is very little data available on the role of other medical conditions in OPC. Individuals with acquired immune deficiency syndrome (AIDS) are known to have an increased risk of all HPV related cancers, and the risk increases with increasing immunosuppression [155]. It has been shown that prevalence of Hepatitis C seropositivity is higher in HPV positive OPC patients compared to control cases with

smoking related cancers (14.0%, (95% CI: 8.7%-19.4%), vs 6.5%, (95%CI:4.6%- 8.3%)), although the mechanisms behind the association are unclear [156].

Moreover, it has been suggested that the reduction in the number of tonsillectomies in the young have influenced the increase of HPV positive OPC [74]. The tonsillar crypt is the likely site of HPV infection and therefore the removal of this tissue may be protective against future tonsillar cancer [75]. This observation does not however explain the increase seen in HPV positive OPC from the base of tongue sub-site over the same time period [74].

In New Zealand, the incidence of OPC is increasing and in 2010-12 period 88% of OPC were HPV positive (Chapter 2) [139]. The cervical cancer vaccine (Gardasil®4) has been offered to girls in New Zealand since 2008, and in 2017 the funded vaccine schedule was expanded to include males and females aged nine to 26 years old with Gardasil® 9 [113]. The vaccine will likely prevent many OPC cases in the future [108], however, these effects are likely to take around three decades to be seen once the vaccinated cohort reaches middle age. Until this time, we are likely to continue to see an increase in HPV positive OPC cases. Thus, the aim of this research was to describe the demographics and exposures of a pre-vaccine population of OPC patients to provide baseline New Zealand data for future comparisons, and to develop a broader understanding of the population at risk for OPC.

4.3 Materials and Methods

4.3.1 Study design

The study was a cross-sectional study of incident oropharyngeal cancer patients from throughout New Zealand. The study was reviewed and approved by the Health and Disabilities Ethics Committee, Northern B (Approval number: 15/NTB/155). The HDEC approval letter is provided in appendix 8. Participants for this study were recruited both prospectively and retrospectively. A flow chart of participant recruitment is shown in Figure 4.1.

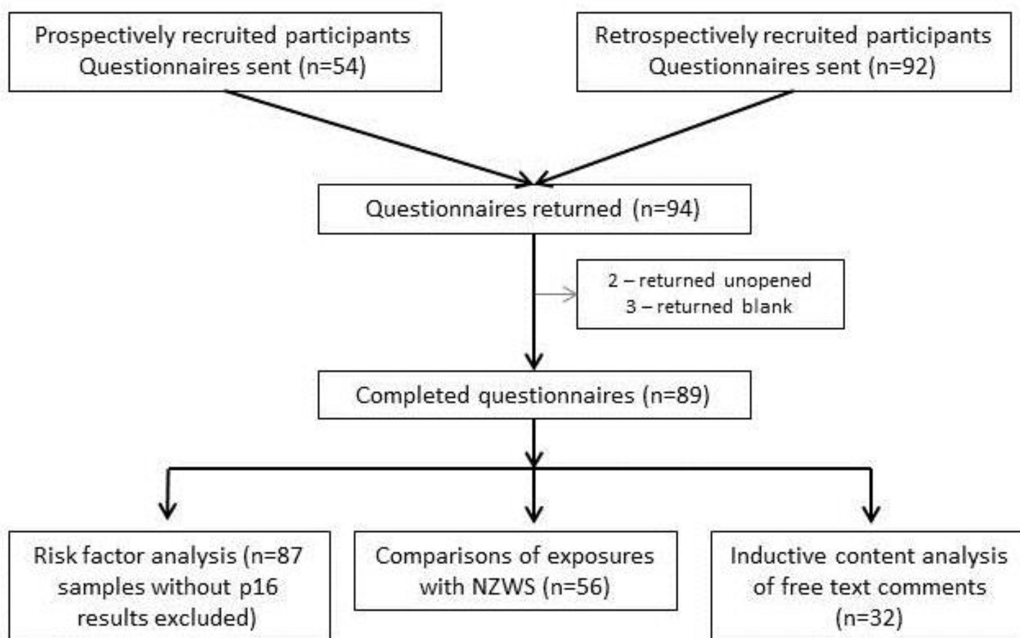

Figure 4.1: Flowchart of participant recruitment and analyses performed.

Prospectively recruited participants were recruited between April 2016 and December 2017. All participants had recently been diagnosed with oropharyngeal cancer and were recruited before commencing radio- or chemo- therapy.

Participants were invited to participate in person at either a scheduled appointment with their Ear, Nose and Throat (ENT) surgeon or at a head and neck multi-disciplinary meeting by the researcher, or a nurse. After recruitment, participants were asked if they would be willing to have tonsillar brushings taken (for use in additional research (Chapter 5)), and answer a questionnaire which would be sent to them within the following month. Written consent was given prior to the collection of the tonsillar brushings. Participants were assigned a unique study identifier and privacy protected by the de-identification of questionnaires and brushings. If completed questionnaires were not received, three reminders were sent to increase the response rate.

Retrospectively recruited patients were participants in previous research who had been diagnosed with oropharyngeal cancer in the time frames; 1996-98, 2003-05 or 2010-12. They had given consent for their stored diagnostic specimen to be used in a previous study and at the time of giving consent had requested a summary of the study's results (Summary received by participants shown in appendix 9). At the same time as the summary of results from previous research was sent out, potential participants (n=92) received a letter (provided in appendix 10) inviting them to complete a modified version of the questionnaire (described below). For participants recruited retrospectively, ethical approval allowed the return of the questionnaire to be taken as consent to participate. No reminder letters were sent for this group.

4.3.2 Sample size calculation

The estimated sample size required for the study was calculated using the variable of ever given oral sex. The calculation was based on the following: We would like to be 80% sure of being able to detect an odds ratio of 4.3 [61] in those reporting having oral

sex, using a 0.05 significance test. We assume that the prevalence of HPV positive OPC in those reporting not having oral sex is 5%, and we expect a ratio of HPV positive to negative cases of 75:25 [130]. Using these figures 148 participants are required. If the assumed prevalence of HPV positive cases in the reference group is increased to 7% the desired sample size becomes 96.

4.3.3 Questionnaire

All participants were asked to complete a four section questionnaire (Questionnaire development is described in Appendix 11, the questionnaire in appendix 12, and the modified questionnaire for retrospectively recruited participants in Appendix 13). Due to the sensitive nature of some questions, all questionnaires were self-administered (either hardcopy or online) at a time and place suitable to the participant. The first section of the questionnaire obtained demographic information, including gender, age at diagnosis, ethnicity, education, income, occupation and exposure to potentially hazardous substances. The second section covered alcohol consumption, both for the 12 months prior to diagnosis and any earlier periods of heavier drinking. Lifetime smoking and marijuana use were also assessed. The third section examined sexual behaviours including the number of partners (both lifetime and before age 25) for both vaginal/anal and oral sex, a history of sexually transmitted infections, and a cervical smear history for females. The fourth section examined the participant's medical history and medication use. Questions included significant medical conditions, if their tonsils had been removed prior to diagnosis, and aspirin, NSAID, and steroid use and lastly if any symptoms were experienced in the six months before their diagnosis.

The wording of the questionnaire for the retrospective patients was modified to fit with the long period between diagnosis and questionnaire delivery.

4.3.4 Laboratory testing

p16 immunohistochemistry (IHC) for prospective cases was performed as part of diagnostic testing at accredited medical laboratories throughout New Zealand. p16 is a tumour suppressor protein and a commonly used surrogate marker for HPV. A positive p16 IHC result relies on the visual detection of brown staining in the nucleus and cytoplasm of at least 70% of tumour cells [86]. The p16 result was given in the pathology report provided for the study. p16 testing on the retrospective cases was completed as part of earlier research (Chapter 2) [139]. Cases were defined as HPV positive or negative based on the p16 result.

4.3.5 Data categorisation and Statistical analyses

Questionnaire responses were entered into a Microsoft access database and analysed using the software package R version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria). Data were re-categorised for analysis.

Ethnicity was categorised as New Zealand European, Māori, or other, with Māori prioritised when multiple ethnic groups were selected. Current occupation was categorised using the Australian and New Zealand standard industrial classification (ANZSIC) with an additional category of “retired” included. Alcohol consumption was reclassified using Ministry of Health low risk drinking advice [157]. Categorisation was based on the multiplication of the days per week drinking and the number of drinks per drinking day to provide a range of drinks per week. A light drinker was equivalent to a low risk drinker and consumed 10 or fewer standard drinks per week for females,

and 15 or fewer for males. A heavy drinker was defined as consuming more than 14 standard drinks a week for females and more than 21 drinks per week for males. As days per week drinking and number of drinks per drinking day were reported in ranges, if the possible range spanned the heavy and light drinker thresholds the person was considered to be a moderate drinker. For tobacco smoking, pack years were calculated using the mean from the selected length of time smoking and mean cigarettes per day results.

4.3.6 Comparisons to the New Zealand Working Survey

The exposures to hazardous substances of the study population were compared to those of the New Zealand Working Survey (NZWS). The NZWS is a study of 3003 men and women aged 20-64 who were selected from the electoral roll and underwent a phone interview. The NZWS questionnaire included questions on: lifetime work history, current exposures, occupational morbidities, and demographic information [158].

4.3.7 Free text comments

Themes in free text comments were identified by inductive content analysis [159,160]. To be defined as a theme a topic must have been described by six or more respondents. Themes were generated independently by two individuals (RL and JB) then compared and a consensus reached.

4.3.8 Uni- and multivariable Analysis

To investigate the association between p16 positive OPC and putative risk factors, each factor was tested individually for significance at $p < 0.2$ in a logistic regression model. A multivariable model was built by a stepwise selection process, retaining all variables

significant at $p < 0.05$. Once a main effects model was built, two-way interaction terms were introduced to the model and retained if significant at $p < 0.05$ and any confounding variables were also included.

4.4 Results

A total of 92 questionnaires were returned between July 2016 and January 2018. Three questionnaires were returned blank with notes as follows: one participant died following chemotherapy complications, one patient no longer wanted to complete the questionnaire, and one participant stated his cancer was not HPV related and therefore deemed the questionnaire irrelevant. Two questionnaires were returned unopened. Overall, 89 completed questionnaires were returned forming the final study population with a response rate of 62%. The response rate for participants recruited prospectively was 87%, and 48% for those recruited retrospectively.

There were 79 p16 positive cases, eight p16 negative cases, and two cases in which p16 testing was not performed. The demographics of the study population are shown in Table 4.1. Raw frequency tables of all questionnaire responses are shown in appendix 12.

Table 4.1: Demographic characteristics of 89 patients with oropharyngeal cancer.

Variable	Level	Number (%)
Recruitment	Prospective	45 (51)
	Retrospective	44 (49)
p16	Positive	79 (89)
	Negative	8 (9)
	Not reported	2 (2)
Gender	Male	69 (78)
	Female	19 (21)
	Missing	1 (1)
Age Group at diagnosis	60 or younger	45 (51)
	61 years or older	44 (59)
Current marital status	Single	21 (24)
	I am married /civil union /de facto partner	60 (67)
	In a relationship	8 (9)
Ethnicity	New Zealand European	72 (81)
	Māori	5 (6)
	Other	10 (11)
	Missing	2 (2)
Highest qualification	None	18 (20)
	High school qualification	21 (24)
	Higher qualification	49 (55)
	Missing	1 (1)

4.4.1 Exposure to hazardous substances

The questions on exposures to hazardous substances were included after the 18 participants were recruited. Of the 18 participants who received these additional questions, four were overall non-responders, and three did not respond to new questions. Exposure to a potentially hazardous substance was reported by 63% (56/89) of participants. It was not asked in the questionnaire if all those reporting occupational exposures had access to appropriate personal protective equipment (PPE). Of the 54 responses to the questions on the use of PPE, 12 said PPE was always used, 21 said sometimes used, and 21 said PPE was not used. Comparison between the demographics and occupations of the NZWS and the study population are shown in Table 4.2. The study population was older, and contained more males than the NZWS. Comparisons between exposures in the study population and the New Zealand working survey are shown in Table 4.3. The study population had significantly more exposures to any hazardous substance, and asbestos and formalin exposure were significantly higher in the study population.

Table 4.2: Comparison of the demographics and occupations of the study population (n=89) and the NZWS (n=3003)

Variable	Level	Study population n (%)	NZWS n (%)	p-value
Gender	Male	67 (78)	1431 (48)	
	Female	19 (21)	1572 (52)	<0.01
Age[1]	< 45	7 (8)	1479 (49)	
	46 – 55	26 (29)	868 (29)	
	>56	56 (63)	656 (22)	<0.01
Ethnicity	NZ European	72 (83)	2454 (82)	
	Maori	5 (6)	279 (9)	
	Other	10 (11)	416 (14)	0.50
Occupation[2,3]	Legislators, administrators, managers and clerical staff	7 (8)	601 (20)	
	Professionals	22 (25)	598 (20)	
	Technicians and Trades workers	8 (9)	511 (17)	
	Service and sales workers	2 (2)	227 (8)	
	Plant & machine operators and assemblers	5 (6)	140 (5)	
	Labourers including agricultural and fishery workers	6 (7)	245 (8)	0.03

[1] Age as per categories used in this study. The NZWS had slightly different age brackets eg. 45-54 where the corresponding bracket in this study was 46-55.

[2] ANZSCO codes from this study and NZSCO codes from the NZWS were combined to form occupation categories. To align the NZSCO codes with the corresponding ANZSCO codes: clerical staff were added to legislators, administers and managers, trades workers were added to technicians, and agricultural and fisheries workers were added to labourers.

[3] Occupation categories of retired (study population) or unemployed (NZWS) were removed.

Table 4.3: Comparison of exposures to hazardous substances between study population (n=89) and New Zealand working survey (n=3003) with 95% confidence intervals for differences and exposures significantly higher in the study population than New Zealand working survey shown by shading.

Substance	Study population Number (%)	NZWS Number (%)	Difference 95% CI
Dust	17 (19.1)	881 (29.3)	-0.173, -0.007
Solvent	13 (14.6)	357 (11.9)	-0.033, 0.116
Pesticides/herbicides/fungicides	12 (13.5)	287 (9.6)	-0.018, 0.126
Fumes	17 (19.1)	984 (32.8)	-0.207, -0.041
Asbestos	6 (6.7)	21 (0.7)	0.024, 0.132
Paints	4 (4.5)	127 (4.2)	-0.026, 0.068
Acids	3 (3.4)	194 (6.5)	-0.055, 0.03
Cement	3 (3.4)	-	-
Welding	3 (3.4)	91 (3.0)	-0.02, 0.064
Chemicals NOS	2 (2.2)	-	-
Cigarette smoke	2 (2.2)	36 (1.2)	-0.006, 0.066
Formalin	2 (2.2)	16 (0.5)	0.001, 0.073
Gas	2 (2.2)	433 (8.5)	-0.143, -0.065
Resins	2 (2.2)	-	-
Timber treatment chemicals	2 (2.2)	69 (2.3)	-0.018, 0.056
Bleach for wool brightening	1 (1.1)	-	-
Condensate class 3	1 (1.1)	-	-

Substance	Study population Number (%)	NZWS Number (%)	Difference 95% CI
Conformal coating of printed circuit boards	1 (1.1)	-	-
Everything involved with tanning a cattle hide	1 (1.1)	-	-
Photographic developer	1 (1.1)	-	-
Styrene	1 (1.1)	-	-
Any substance	56 (62.9)	1488 (49.6)	0.028, 0.228

4.4.2 Alcohol, Tobacco and marijuana use

Descriptions of the alcohol, tobacco, and marijuana use of study participants are shown in Table 4.4. When looking at alcohol consumption in the 12 months prior to diagnosis seven participants did not drink alcohol, 80% (71/89) of the population were low or moderate drinkers and 12% (11/89) were heavy drinkers. In terms of lifetime alcohol consumption, 78% (69/89) of participants had a period of heavier drinking when younger. When lifetime and pre- diagnostic drinking patterns were both considered almost half the study population (40/89) were considered heavy drinkers at some point in their life.

The majority of study participants (72/89) were non-smokers in the 12 months prior to their diagnosis. However, over half the study population (48/89) had been a regular smoker at some point in their lives.

A total of 48 participants had ever used marijuana. Of these 40% (19/48) were considered only experimental users as they had used the drug less than five times in their life. When marijuana use was stratified by p16 status none of the p16 negative cases had ever used marijuana compared to 58% (46/79) p16 positive cases. The remaining two cases reporting marijuana use were those without a p16 result available.

Table 4.4: Description of alcohol, tobacco, and marijuana use of 89 oropharyngeal cancer patients.

Variable	Level	Number (%)
Alcohol consumption 12 months before diagnosis	Do not drink alcohol	7 (8)
	Light	58 (65)
	Moderate	13 (15)
	Heavy	11 (12)
Binge drinking for 12 months prior to diagnosis	No	56 (63)
	Yes	33 (37)
Period of more drinking	No	20 (22)
	Yes	69 (78)
What age during heavier drinking[1]	Under 20 years old	24 (35)
	21 to 25 years old	40 (58)
	26 to 30 years old	30 (43)
	Over 31 years old	24 (35)
Life time ever a heavy drinker	No	49 (55)
	Yes	40 (45)

Variable	Level	Number (%)
Current smoker	No	72 (81)
	Yes	17 (19)
Ever smoked	No	40 (45)
	Yes	48 (54)
	Missing	1 (1)
Pack years [2]	0-5	18 (38)
	6-10	6 (13)
	11 or more	23 (48)
	Missing	1 (1)
Ever used marijuana	No	41 (47)
	Yes	46 (51)
	Missing	2 (2)
How long for [3]	less than 5 times in my life	19 (41)
	Less than 2 years	5 (11)
	2 to 4 years	6 (13)
	5 to 10 years	5 (11)
	11 to 20 years	6 (13)
	Greater than 20 years	5 (11)
How often [4]	A few times a year	7 (26)
	Once or twice a month	8 (30)
	Once or twice a week	11 (41)
	Almost every day	1 (3)

[1] calculated as percentage of the 69 participants who reported a period of heavier drinking, percentages total >100 as multiple age ranges were able to be selected.

[2] calculated as percentage of the 48 participants who reported ever being a regular smoker.

[3] calculated as percentage of the 46 participants who reported ever using marijuana.

[4] calculated as percentage of the 27 participants who reported more than experimental use of marijuana.

4.4.3 Sexual behaviours

Around two thirds of the study population (61/89) were aged 16-19 years old at sexual debut (Table 4.5). The majority of the study population (60/89) had six or more lifetime sexual partners. When lifetime sexual partners were split by p16 status, no p16 negative case had more than 20 partners, compared to 24% (19/79) of p16 positives cases. When looking at oral sex the age at debut appeared slightly older with 45% (40/89) of participants having had oral sex after age 20. Condom use before 25 was low in the study population with only one participant reporting they always used condoms, and 30% (27/89) reporting never using condoms.

Table 4.5: Description of sexual behaviours and sexual health of 89 oropharyngeal cancer patients.

Variable	Level	Number (%)
Age at debut	15 years old, or younger	14 (16)
	16 to 19 years old	61 (68)
	20 years old, or older	13 (15)
	Missing	1 (1)
Lifetime number of partners	1 or 2 partners	8 (9)
	3 to 5 partners	20 (22)
	6 or more partners	60 (68)
	Missing	1 (1)
Partners before age 25	1 or 2 partners	22 (25)
	3 or more partners	63 (71)
	Missing	4 (4)
Oral sex age at debut	15 years old, or younger	5 (6)
	16 to 19 years old	32 (36)
	20 years old, or older	40 (45)
	I have never had oral sex	9 (10)
	Missing	3 (3)
Oral sex lifetime partners [1]	1 or 2 partners	21 (27)
	3 or more partners	55 (72)
	Missing	1 (1)
Oral sex partners before age 25 [1]	1 or 2 partners	36 (47)
	3 or more partners	36 (47)
	Missing	5 (6)

Variable	Level	Number (%)
Condom use before age 25	I never used condoms	27 (30)
	I rarely used condoms	28 (32)
	I sometimes used condoms	21 (24)
	I usually used condoms	11 (12)
	I always used condoms	1 (1)
	Missing	1 (1)
Ever had STI	Yes	33 (37)
	No	55 (62)
	Missing	1 (1)
Had cervical smear in last 5 years[2]	Yes	14 (50)
	No	5 (50)
Had an abnormal cervical smear[2]	Yes	5 (26)
	No	14 (74)
Age at time of abnormal smear [3]	Under 25 years old	1 (20)
	26 to 35 years old	4 (80)

[1] calculated as percentage of the 77 participants who reported ever having oral sex.

[2] calculated as percentage of the 19 female participants.

[3] calculated as percentage of the 5 female participants who reported having an abnormal smear.

4.4.4 Medical history

Table 4.6 summarises the medical histories of study participants. Tonsillitis was the most commonly reported medical condition, experienced by 53% (47/89) of participants. Gastric reflux was experienced by 28% (25/89) of participants. The other medical conditions were varied and included six participants who had had cancer at another body site.

Table 4.6: Summary of medical histories of 89 oropharyngeal cancer patients.

Variable	Level	Number (%)
Suffered from a medical condition	Tonsillitis	47 (53)
	Glandular fever	17 (19)
	Gastric Reflux	25 (28)
	Type 1 diabetes	1 (1)
	Type 2 diabetes	7 (8)
	Rheumatoid arthritis	3 (3)
	Asthma (requiring an inhaler)	11 (12)
	Autoimmune disease	1 (1)
	Heart disease	11 (12)
	Rheumatic fever	3 (3)
	Cancer at another body site	6 (7)
	Other significant medical condition	19 (21)
Had tonsils removed	Yes	27 (30)
	No	58 (65)
	Missing	4 (5)

Variable	Level	Number (%)
Had any in/around throat	Severe injury or trauma	2 (2)
	Surgery	7 (8)
	Chemotherapy or radiation	4 (5)
Had an organ transplant	Yes	1 (1)
	No	80 (90)
	Missing	8 (9)
Aspirin use in last 12 months	Daily	8 (9)
	1 to 6 times a week	2 (2)
	Less than once a week	20 (2)
	Not taken in the last 12 months	57 (64)
	Missing	2 (2)
NSAID use in last 12 months	Daily	6 (7)
	1 to 6 times a week	9 (10)
	Less than once a week	37 (42)
	Not taken in the last 12 months	32 (35)
	Missing	5 (6)
Period of heavier aspirin or NSAID use	Yes	28 (31)
	No	60 (68)
	Missing	1 (1)
Drug used during heavier use[1]	Aspirin	5(18)
	NSAID	23(82)
Prescribed steroid drugs	Yes	30(34)
	No	57(64)
	Missing	2 (2)

Variable	Level	Number (%)
Ever suffered from allergies	Yes	28 (31)
	No	60 (68)
	Missing	1 (1)
Type of allergy[2]	Food	6 (21)
	Drug	4 (14)
	Hayfever	14 (50)
	Eczema	7 (25)

[1] calculated as percentage of the 28 participants who reported a period of heavier aspirin or NSAID use.

[2] calculated as percentage of the 28 participants who reported suffering from allergies, percentages total >100 as some participants suffered from more than one allergy type.

4.4.5 Symptoms

The most common symptom experienced by participants in the six months prior to their diagnosis was having a lump in the neck (Table 4.7). Other symptoms reported by patients were varied and included two reports of bad breath, and two of sleep apnoea.

Table 4.7: Description of symptoms experienced by study participants in the six months prior to their diagnosis.

Symptom	Number (%)[1]
Difficulty or pain in swallowing	15 (17)
Difficulty in opening mouth and/or jaw	4 (5)
Hoarseness of the voice	13 (15)
Non-healing lesions of the mouth	5 (6)
Unusual bleeding in the mouth and/or throat	2 (2)
A feeling of something being ‘stuck’ in the throat	17 (19)
Earache	16 (18)
A persistent sore throat	20 (22)
An unusual smelling body odour/sweat	3 (3)
A lump/s in the neck	43 (48)
Other	22 (25)

[1] percentages total >100 as multiple symptoms were able to be selected.

4.4.6 Uni- and multi-variate analysis

The univariate analysis of putative risk factors showed method of recruitment ($p = 0.17$), gender ($p = 0.06$), age group ($p = 0.15$), sexual partners before age 25 ($p = 0.11$) and ever given oral sex ($p < 0.01$) to be significant (Table 4.8). The following factors were also examined and found to be non-significant: marital status, ethnicity, education, current drinking risk, previous drinking risk, smoking, age at sexual debut, and having had another STI, In the final multivariable model (Table 4.9), there was a statistically significant association between p16 result and ever having given oral sex (OR: 15.86, 95% CI: 1.35-230.54, ever vs never). The variable of ever given oral sex was strongly confounded by the number of sexual partners before 25. This confounding is shown by the reduction in the univariate OR for ever given oral sex from 30.0 to 13.0 when the number of sexual partners before 25 is added to the model.

No interactions were found between ever given oral sex and gender, age group, and the number of sexual partners before age 25. However, when checking for interaction between ever given oral sex and recruitment, the inflated standard errors indicated data scarcity and a lack of convergence in the model.

Table 4.8: Univariate analysis of putative risk factors associated (p≤0.2) with having a p16 positive tumour.

Variable	n	level	p16 positive	p16 negative	OR (95% CI)	P value
Recruitment	87	Prospective	38	6	REF	
		Retrospective	41	2	3.23 (0.70-23.02)	0.17
Gender	86	Female	15	4	REF	
		Male	63	4	4.20 (0.90-19.70)	0.06
Age group	87	60 and under	42	2	REF	
		61 and over	37	6	0.29 (0.04-1.36)	0.15
Sexual partners before age 25	83	1 or 2	19	3	REF	
		3 or more	59	2	4.66 (0.72-37.41)	0.11
Ever given oral sex	84	No	5	4	REF	
		Yes	73	2	29.20 (4.62-254.02)	<0.01

Table 4.9: Multivariable analysis (n=80) of putative risk factors associated with having a p16 positive tumour.

Variable	level	OR (95% CI)	P value
Ever given oral sex	No	REF	
	Yes	15.67 (1.34-227.62)	0.03
Recruitment	Prospective	REF	
	Retrospective	7.33 (0.60-269.44)	0.17
Gender	Female	REF	
	Male	5.45 (0.46-74.98)	0.17
Age group	60 and under	REF	
	61 and over	0.96 (0.07-11.90)	0.98
Sexual partners before age 25	1 or 2	REF	
	3 or more	2.93 (0.22-44.62)	0.40

4.4.7 Inductive content analysis

Analysis of the free text comments (comments provided in appendix 13) revealed two themes. The first theme was factors participants felt contributed to their cancer. There were 16 comments around such contributors. Within this theme the self-identified contributing factors were either: work exposures (n = 7) including factors such as asbestos, welding fumes, and the use of fly spray in butcheries. Lifestyle exposures (n = 6) including sugar intake, and the use of alcohol based mouthwash. Or personal exposures (n = 3) including major personal stress such as the death of a close relative.

The second theme was the personal recognition of symptoms (n= 11). This included comments such as “an increased gag reflex when cleaning teeth” and “earache symptoms were mild and the sore throat I originally thought was part of a cold”.

There were three comments around the delayed recognition of disease by a medical professional, although this fell below the threshold for a theme.

4.5 Discussion

This is the first study to describe a pre-vaccine group of oropharyngeal cancer patients in New Zealand. Of the participants in this study, 91% had p16 positive OPC. This finding is consistent with national data, as in 2010-12 88% of OPC were HPV positive (Chapter 2) [139]. This high proportion of p16 positive cases in a small study population led to limitations in some analyses due to data scarcity. However, results from the multivariable model found ever having given oral sex to be significantly associated with having a p16 positive tumour and consistent with international data

[61,63]. For example, in a case control study of 240 head and neck cancer (HNSCC) cases and 322 controls, Gillison *et al* found having 1-5 lifetime oral sex partners to be associated with an HPV positive tumour (OR 2.7 (95% CI: 1.0-7.5)). This association increased in strength with the number of partners with an OR of 5.6 (95% CI: 1.8-17.7) for 15 or more lifetime oral sex partners [63].

Associations between marijuana and HPV have previously been described in overseas studies [63,69]. These associations increased with increasing intensity of use and were corrected for tobacco and alcohol use [63,69]. This study found almost half of the participants with p16 positive OPC had used marijuana compared to no participants with p16 negative tumours. However, further analysis on the use of marijuana was not possible due to data scarcity.

The inclusion of retrospective and prospective participants may have introduced some recall bias in our study. Prospective patients were either about to start or were undergoing therapy at the time of completing the questionnaire. By comparison retrospective participants had between five and 20 years since their diagnosis. The importance of an event in one's life and the associated emotion can impact on the ability to recall information, and may also lead to overestimation of exposures [161]. It is described that head and neck cancer is the most emotionally traumatic of all cancers, due to the impact of the cancer, and the associated treatment, on appearance and function of the upper aero-digestive tract [55]. It is possible that prospective participants were under more emotional stress at the time of completing the questionnaire, and therefore may have over-reported some factors. It is also possible

that any retrospective participants with long term morbidities due to their cancer may have caused a similar effect.

Questions seven to ten in our questionnaire on occupation, exposures to hazardous substances, and the use of PPE (see appendix 11) were included after participant recruitment had begun and after conversations with and feedback from participants. Early discussions with participants at recruitment revealed three participants that were welders. They all made comment to the inhalation of welding fumes as a possible contributor to their cancer. Welding fumes have previously been linked to an increased risk of lung cancer [162].

This study compared the exposures of participants to the New Zealand working survey [158]. The NZWS had an approximately equal number of males and females, and an average participant age of 43.5 years, as expected when using a population of oropharyngeal cancer patients, our study population was older, and predominantly male. There was a significant difference between occupations in the NZWS and our study population (p=0.03). The major differences were the lower proportion of those in legislative/administration/clerical positions (8% versus 20%), and technician/trade roles (9% versus 17%%)in our population compared to the NZWS population. The study population also had an additional 22 participants who were retired and therefore had no current occupation. It is likely excluding these individuals, which made up approximately a quarter of the study population contributed to this difference in occupations. When looking at the previous occupations the retired participants engaged in, seven had occupations in the legislators/administration/clerical category and eight in the technicians or trades workers categories. Knowing this additional

background occupational information improves the value of comparison between the studies.

Exposures in the NZWS study were self-reported. Our questionnaire collected data on the length of each exposure however; due to the low numbers in our study, analysis was limited to the type of substance only, without duration of exposure.

This study used self-reported exposures to hazardous substances. However, studies by Paget-Bailly *et al* and Peng *et al* estimated exposures based on job matrices [153,154]. This approach was not possible in our study due the large number of individuals who were retired, both at the time of diagnosis and at study recruitment, and also the limited information we had on previous employment. It is possible some exposures are under- or over-represented. We found a significantly higher exposure to formalin and asbestos, and a significantly lower exposure to fumes, dust and gas in our population compared to the NZWS. The difference in these exposures may be affected by comparing exposures of cancer patients to those of a healthy population. It is possible those who have had cancer are more likely to recall exposures to well known carcinogens such as asbestos, and less likely to recall exposures to more common factors such as dust or fumes. There are differences between the NZWS and the study population, and because of these the differences seen in exposures should be interpreted with caution. However, the potential role of asbestos and/or formalin in OPC has some plausibility and this is described below.

Formaldehyde has a wide range of industrial uses and is classified as a human carcinogen by the International Agency for Research on Cancer (IARC) [152]. This study found oropharyngeal cancer patients were significantly more exposed to formalin than

the New Zealand working population. A study of workers in formaldehyde industries covering 998,239 person years found a relative risk of 7.66 (95% CI: 0.94-62.34) for nasopharyngeal cancer in those with the highest exposure to formaldehyde [152]. Another study of occupational exposures and the risk of hypopharyngeal/laryngeal cancer suggested a positive association between formaldehyde exposure and laryngeal cancer [151]. Formaldehyde is a known animal carcinogen and inhalation can induce nasal cancer in rats [163]. However, there is limited evidence that inhaled formaldehyde reaches past the nasal tissue in either rats or humans [163]. Interestingly, in the above study of workers in formaldehyde industries five out of the ten observed nasopharyngeal cancer deaths were from one plant, with the remaining nine plants experiencing a lower than expected incidence of nasopharyngeal cancer [152]. It is possible confounding exposures existed, including exposures to other known human carcinogens including solvents and wood dust [163].

Asbestos was widely used in industry until the 1970s, when relationships between asbestos exposure and mesothelioma and lung cancer were described [154]. Subsequently, associations between asbestos exposure and head and neck cancers have also been described [151,153]. The most well studied head and a neck cancer is the association between asbestos exposure and laryngeal cancer. A systematic review and meta-analysis of 21 studies found a standardised mortality ratio of 1.69 (95% CI: 1.47-1.97) for laryngeal cancer patients exposed to asbestos [154]. In a study of 1833 HNSCC patients and 2747 controls, Paget-Bailly *et al* found an odds ratio of HNSCC in those exposed to asbestos of 1.9 (95% CI: 1.6-2.3). When HNSCC was split by anatomic sub-site, the odds ratio for OPC in those exposed to asbestos was 1.6 (95% CI: 1.3-2.1) [153].

This study presents a description of exposures and demographics of a pre-vaccine population of OPC patients. The increased occupational exposures highlight the need for further research on the potential role of occupational exposures in OPC, assessing both specific industries and individual substances. Further research into possible interactions between HPV and substances, particularly those that can be inhaled is also required. Inductive content analysis has also provided an insight into patient's views on what may have contributed to their cancer, and these views highlight the need for increased public education and awareness about the role of HPV in cancer.

Chapter 5 - HPV 16 mRNA and DNA detection, viral load, and cytology in tonsillar brushings from conscious oropharyngeal cancer patients.

5.1 Abstract

Background and aims: Detection of precancerous lesions forms the base of cervical cancer screening, which has seen dramatic reductions in the incidence and mortality from Human Papillomavirus (HPV)-induced cervical cancer. However, precancerous lesions for HPV positive oropharyngeal cancer are not well described and there is limited research on a minimally invasive pap-test equivalent for oropharyngeal cancer. The aim of this study was to use brushings from conscious oropharyngeal cancer patients to investigate the detection of HPV 16, and cytological abnormalities.

Methods: Brushings were taken from the tumour site and/or an adjacent site (e.g. a contralateral tonsil). p16 results were provided in diagnostic pathology reports. A liquid based cytology preparation was made and a qPCR for the detection of HPV 16 performed on all specimens. On HPV 16 DNA positive specimens, the viral load per copy of human beta-globin was calculated and RNA extracted in order to detect transcriptionally active virus.

Results: Seventy seven brushings were collected from 54 patients, and of these 81% were p16 positive. The risk of HPV 16 DNA detection and abnormal cytology in brushings taken from an abnormal appearing site were 3.0 (95% CI: 1.4-6.3) and 4.0 (95% CI: 1.5-10.3) times greater respectively than the risk from a normal appearing brushing site. Cellular changes comparable to cervical precancerous lesions were present in 27 samples. Eight samples showed a continuum of dysplastic change and all were from p16 positive samples.

Conclusions: These results show the detection of viral DNA and cytological abnormalities is possible in brushings from conscious patients. The cellular changes comparable to cervical precancerous lesions warrant further investigation.

5.2 Introduction

Human papillomavirus (HPV) positive oropharyngeal cancer (OPC) is a distinct entity from traditional tobacco and alcohol associated head and neck cancers, and tends to affect younger patients who are non-smokers and light drinkers, with the incidence being higher in males [41]. The major risk factor for HPV positive OPC is sexual behaviour including the number of sexual partners and practising oral sex [41,61]. The increase in incidence of HPV positive OPC in developed countries has been described as a “virus related cancer epidemic” [49]. The increase began in the 1970s and the incidence continues to rise. Many countries, including the United States, are likely to see the number of HPV attributable oropharyngeal and cervical cancer cases equilibrate in the near future, thus starting to balance the burden of HPV related cancers between the sexes [46]. Ministry of health data shows in New Zealand in 2012 there were 116 cases of squamous cell carcinoma of the cervix, and 126 oropharyngeal cancer cases, with 88% of these likely to be HPV positive [139]. This data shows the above equilibrium is also likely to be reached in New Zealand.

The prophylactic vaccine Gardasil® is likely to have significant impact on the incidence of HPV positive OPC in the future provided adequate coverage is achieved [108]. Although not specifically designed for oral HPV infection, the Costa Rica vaccine trials have calculated a vaccine efficacy of 93.3% based on HPV 16/18 detection on oral

rinses collected four years post vaccination [120]. Gardasil®4 has been available in New Zealand since 2008 and was initially funded for females born after 1990. From early 2017, Gardasil®9 was introduced in New Zealand and the funded vaccination schedule expanded making the vaccine free to males and females aged 9-26 years of age [164]. The effects of vaccination on HPV positive OPC incidence will take decades to be seen, and until those who are vaccinated reach middle age HPV positive OPC cases will be seen in a pre-vaccine population. This population would benefit from the development of secondary and tertiary prevention strategies [165].

There are similarities between cervical and HPV positive oropharyngeal cancers, such as a long time between initial infection and the development of cancer, as shown by the detection of E6 antibodies up to 10 years before OPC diagnosis [77]. However, there are a number of factors that limit secondary prevention strategies. Firstly, precancerous lesions for OPC have been rarely detected and are not well described [43,81]. This may be explained by genital HPV infection being more common than oral HPV infection [43]. For example, a study of 3377 fixed and 511 fresh frozen tonsillectomy samples had no cases positive for the presence of HPV DNA by PCR [166]. By comparison, the overall HPV prevalence by PCR in cervico-vaginal specimens from females aged 14-59 was 42.5% [31]. Of note, histopathological examination of tonsillar cancers has shown dysplasia adjacent to HPV positive tumours in less than five percent of cases [81]. This may suggest that although HPV infections in the tonsil are less frequent than in the cervix, the proportion of infections that progress to cancer may be higher [166].

Secondly, the cervical transformation zone is the site of HPV infection and is a flat surface relatively accessible for sampling [124]. In contrast, the site of HPV infection in the tonsils is within the depths of the crypts which are technically difficult to sample [124,165]. Brushings taken from the tumour surface of head and neck cancer patients under anaesthetic having panendoscopy have shown to have a sensitivity and specificity for HPV detection of 83% and 85% respectively [125]. HPV DNA detection has been associated with oropharyngeal cancer in cases under investigation for oropharyngeal abnormalities, however, when the same brush based test was applied to an at risk population with no visible lesions, no associations between HPV DNA detection and abnormal cytology were found [123]. The crypt epithelium of the tonsils contains both squamous cells and lymphocytes. The masking of squamous cells by numerous lymphocytes can limit the utility of brush based tests, in particular those for cytological evaluation [127]. In order to overcome these technical difficulties, an oral rinse has been used as an alternative to brush based tests [126,167]. However, the usefulness of this technique is limited as there is no evidence that HPV infection in the oropharynx can be inferred from a positive oral rinse [126,166]. In addition, HPV detection rates are consistently higher in oral rinses than brushings taken from head and neck cancer patients, with the detection of HPV at the tumour site showing better correlations with cancer risk, than detection in the rinse sample [167].

Finally, the introduction of any screening test must have clearly defined benefits that outweigh the harms [168]. There is currently no acceptable screening test to reliably identify those at risk [123], and it is unknown if early detection of HPV positive OPC would lead to reduced mortality from the disease [108]. Before further work is undertaken on the role of screening in OPC, more work is required on whether

minimally invasive sampling techniques can reliably detect clinically significant HPV infection in the oropharynx.

Thus, the aims of this research were to trial the use of brushings from conscious OPC patients and to investigate the detection of HPV 16 DNA, RNA, and viral load, and the presence of any cytological abnormalities.

5.3 Materials and Methods

Ethics approval was obtained from the Health and Disabilities Ethics Committee (reference: 15/NTB/155). Participant privacy was upheld by de-identification of samples.

5.3.1 Participant recruitment and sample collection.

A flow chart of participant recruitment and specimen testing is shown in Figure 5.1. Potential participants for this study were patients diagnosed with oropharyngeal cancer between April 2016 and December 2017, who had not commenced radio- or chemotherapy. The participant information sheet/consent form is shown in appendix 14. Participants had brushings taken in a clinic setting by an Ear, Nose and Throat (ENT) surgeon, nurse or researcher, or in theatre. The brushing site/s and appearance of the area were recorded. Brushings were taken using a cytobrush (Thermo Scientific, Waltham, USA), with one cytobrush and vial used per brushing site. Cytobrushes were then vigorously rinsed in 5 mL PreservCyt (Hologic, Marlborough, USA). Brushings sites were defined as of the tumour, an adjacent site (the contralateral tonsil or base of tongue) or a distant site (in metastatic tumours). All cases had been diagnosed with

oropharyngeal cancer within the month prior to recruitment and diagnostic pathology reports including p16 results were provided on entry into the study.

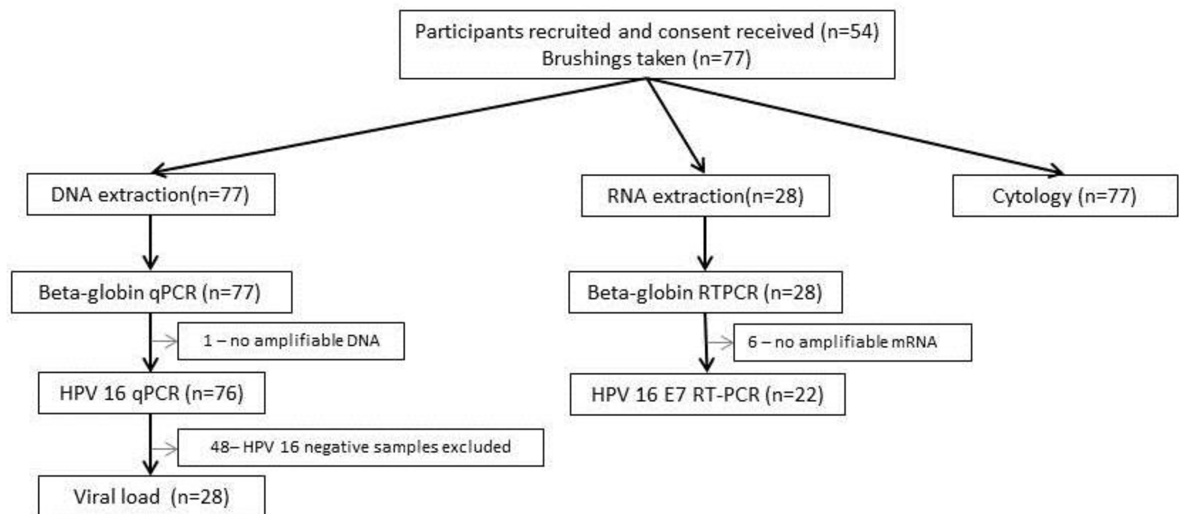

Figure 5.1: Flowchart of participant recruitment and testing performed.

5.3.2 DNA extraction

After vortexing to resuspend cellular debris, 1 mL of sample was used for DNA extraction. Samples were centrifuged at 20,000 g for five minutes, the supernatant removed, and pellet re-suspended in 200 µL PBS. DNA extractions were performed using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany). A control extraction containing only PBS and no sample was included with each set of study samples. The control extractions were then used as negative controls in the beta-globin and HPV 16 qPCRs. A final elution volume of 100 µL was used. The quality and quantity of extracted DNA was assessed using a Nanodrop™ Spectrophotometer (Thermo Scientific). Extracted DNA was stored at -20 °C until processing.

5.3.3 RNA extraction

RNA extractions were performed on samples positive for HPV 16 DNA. After vortexing to resuspend cellular debris, 1 mL of sample was used for RNA extraction. RNA extractions were performed using the RNeasy® Minikit (Qiagen). Samples were centrifuged at 20,000 g for 5 minutes, the supernatant removed, and pellet re-suspended in 350 µL Buffer RLT . An on column DNase digestion was included and a final elution volume of 40 µL was used. A control extraction containing only RLT buffer and no sample was included with each set of study samples. The control extractions were then used as negative controls in the beta globin and HPV 16 qPCRs. The quality and quantity of extracted RNA was assessed using a Nanodrop™ Spectrophotometer (Thermo Fisher Scientific). Extracted RNA was stored at -80 °C until processing.

5.3.4 Beta-globin qPCR

The presence of amplifiable genomic nucleic acid was assessed on all cases by qPCR targeting the human beta-globin gene using the PC03 and PC04 primers as previously described [139]. A sample was considered positive if it crossed the threshold and produced a T_m of 81.0°C (±1.0°C) after 40 cycles. Duplicate standard curves from seven, 10-fold serial dilutions starting from 2.03 x 10^6 copies of the beta-globin clone control (described below) were used to determine the limit of detection.

5.3.5 HPV 16 E7 qPCR

The presence of HPV 16 E7 was assessed on all cases with a positive beta-globin qPCR DNA result. A qPCR with melt curve analysis was developed using previously described primers and cycling conditions [143]. Each reaction mix contained: 0.15 µM each primer and 1X Fast Start SYBR Green Master (Roche, Basel, Switzerland).

Approximately 30 ng of extracted nucleic acid was used as template. PCR was followed by a melt curve from 70 – 80°C with 0.2°C increments and a five second hold. A sample was considered positive if it crossed the threshold and produced a T_m of 78.5°C (±1.0°C). Duplicate standard curves from six, 10-fold serial dilutions starting from 6.92 x 10^5 copies of the HPV 16 clone control (described below) were used to determine the limit of detection.

5.3.6 RT-PCR

Before cDNA synthesis extracted RNA was subject to the HPV 16 qPCR, as described above, to check for contaminating DNA. If DNA was present an additional DNase treatment was performed. cDNA was synthesized using the First Strand Transcriptase cDNA synthesis kit (Roche). Reactions were as per manufacturer's instructions. cDNA was used in the beta-globin and HPV 16 E7 qPCR's described above.

5.3.7 Viral load calculations

Viral load on HPV 16 E7 DNA positive samples was calculated per copy of the human beta-globin gene. A positive beta-globin control was made from cloned DNA of a study sample. Cloning was performed using a pGEM®-T Easy kit with JM109 High Efficiency Competent Cells (Promega, Madison, USA), according to the manufacturer's instructions with blue/white selection. The cloned plasmid contained an approximately 408 bp fragment of the human beta-globin gene sequence amplified using the GH20 and GH21 primers [133]. Before being used as a positive control, the cloned plasmid was subjected to automatic dye-terminator cycle sequencing with BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyser (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA)

to confirm genomic sequence using both the forward and reverse primers. The sequences obtained were compared using the NCBI Blast database to other published sequences available from GenBank [135]. The HPV 16 positive control was a previously described cloned plasmid that contained an approximately 1.8 kb fragment of the HPV 16 genome that included the E6 and E7 gene sequence [139].

Copy numbers for HPV 16 and beta-globin were determined in duplicate by using standard curves, generated in the same PCR run with either the HPV 16 or beta-globin plasmid. Standard curves were made from six 10-fold serial dilutions with the beta-globin curve starting at 2.03×10^6 copies, and the HPV 16 curve starting at 6.92×10^5 copies. Viral load was calculated by dividing the mean HPV 16 copy number by the mean beta-globin copy number. HPV DNA load was defined as the number of HPV DNA copies per beta-globin gene copy.

5.3.8 Liquid based cytology preparations

Liquid based cytology (LBC) preparations were made on all cases. After vortexing to resuspend cellular debris, 1 mL of sample was transferred to a ThinPrep® vial (Hologic). Vials were stored at room temperature until processing. Cytology specimens were prepared using the ThinPrep® 2000 processor, and slides stained with the ThinPrep® Stain on an autostainer (Leica Biosystems, Wetzlar, Germany). All slides were screened blind by two experienced cyto-scientists (RL & SY), and all slides with an abnormality seen by either screener were read independently by two pathologists (BL and CT), and discordant cases reviewed to reach a consensus. Cytological abnormalities were classified using the Bethesda system for cervical cytology, then further categorised to unsatisfactory, normal, low grade, high grade or malignant.

5.3.9 Statistical analysis

Initial exploration of data was by summary statistics, tables and plots. The following associations were explored using two by two tables: test results and brushing site; test results and appearance; and between different test results. Risks and p-values were calculated for each association. Viral load was transformed using the natural log transformation. Descriptive analysis was performed using the software package R version 3.2.0 (R Development Core team, 2010, R Foundation for Statistical Computing, Vienna, Austria).

5.4 Results

5.4.1 Sample collection.

A total of 77 brushings from 54 patients were included in this study, with one to four brushings being taken per participant. Brushings were collected from patients under anaesthetic in six cases, conscious patients in 46 cases, and the patient state was not recorded in the remaining two cases. All but one case had an available p16 result with 81% of cases (44/54) being p16 positive. Brushings were taken from the tumour site in 47% of cases (36/77), an adjacent site in 45% (35/77), and a distant site in the remaining six samples. The brushing site appeared normal in 40% (31/77) of samples. Of the 30 brushings taken from an abnormal appearing site, 13 brushings were taken from a visible tumour. The appearance of the site was not recorded for the remaining 16 brushings.

5.4.2 HPV 16 E7 DNA detection

Serial dilutions of a beta-globin clone control showed the assay was capable of detecting a minimum of 13 copies of the target sequence. Following the beta-globin PCR, one sample was excluded due to a lack of amplifiable human DNA. Serial dilutions of the HPV 16 clone control showed the assay was capable of detecting nine copies of the HPV 16 E7 target sequence.

HPV 16 DNA was detected in 37% (28/76) of brushings. Of the HPV 16 positive samples, 96% (27/28) were in p16 positive samples. HPV 16 positive brushings were from the tumour site in 79% (22/28) samples, with three positive samples from an adjacent site, and three from a distant site. Of the HPV 16 positive samples, the appearance of the brushing site was abnormal in 19, within normal limits in six and not recorded in three samples. There were 12 p16 positive cases that had the appearance noted as visible tumour. Of these 83% (10/12) were positive for HPV 16 DNA. The risk of HPV 16 DNA detection in brushings taken from an abnormal appearing site was 3.0 (95% CI: 1.4-6.3) times greater than the risk of HPV 16 DNA detection in brushings taken from a normal appearing brushing site.

5.4.3 Viral load

Calculated viral loads ranged from 0.01 to 70.34 copies of HPV 16 E7 per human beta-globin copy, with a median viral load of 1.27 copies, and mean of 10.15 copies. The associations between the log of viral load and cytology result, mRNA detection, and the appearance of the brushing site are shown in figure 5.2. Although there were no statically significant associations, the general trend was viral load increased with

increasing levels of cytological abnormality, was higher in HPV 16 mRNA positive samples, and a higher in samples taken from an abnormal appearing brushing site.

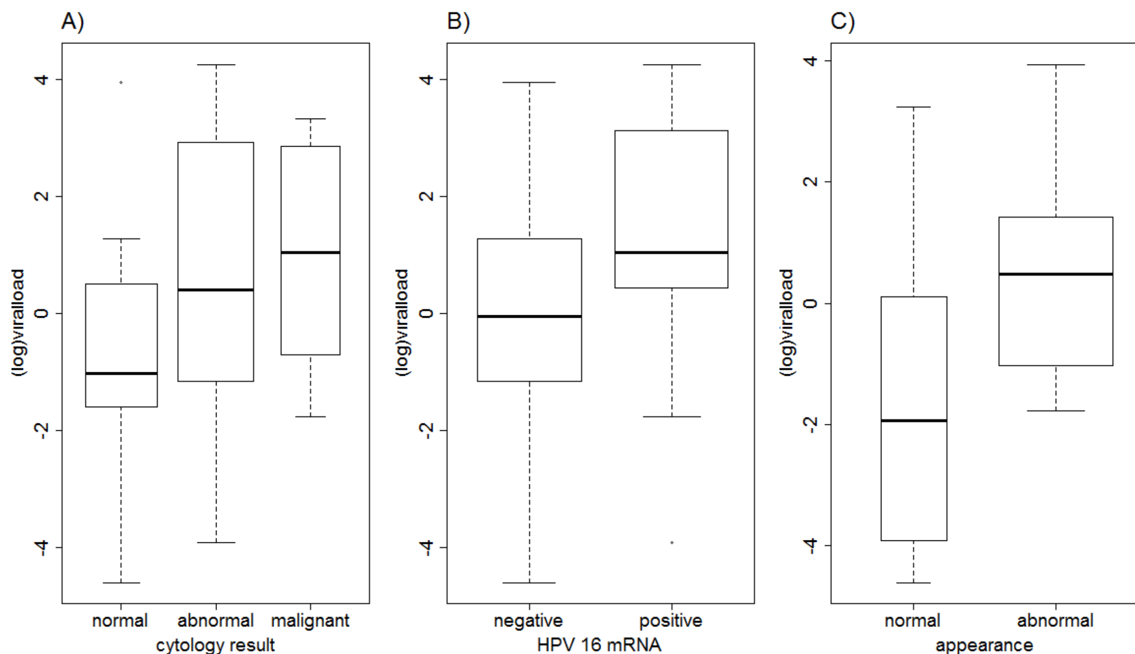

Figure 5.2: Boxplots of viral loads from brushing samples. Relationship of (log) viral load and A) cytology results, B) mRNA detection and C) brushing site appearance.

5.4.4 HPV 16 E7 RNA detection

Following the beta-globin RT-PCR, six samples were excluded due to lack of amplifiable DNA. HPV 16 RNA was detected in 55% (12/22) of samples. All samples with detectable RNA were taken from the tumour site, and 75% (9/12) had an abnormal appearance. The brushing site appeared normal in one case, and was not recorded in the final two cases.

5.4.5 Liquid based cytology

Cytology results were unsatisfactory in 8% (6/77) of cases, three for insufficient squamous cells, two for blood obscuring the cells and one for both insufficient cells and obscuring blood. There were normal cytology results in 58% (45/77) of cases, and of these eight showed reactive or inflammatory changes. There were organisms present consistent with *Candida* species in two cases, and cellular changes consistent with herpes simplex virus infection in a further two cases. Of the 26 cases with abnormal cytology, six showed invasive squamous cell carcinoma (SCC), a further 12 cases showed high grade abnormalities: four with high grade dysplasia where invasion could not be excluded (HS2), six with high grade dysplasia (HS1), and two with atypical squamous cells where high grade could not be excluded (ASH). Low grade (LS) changes were seen in eight samples, with five showing low grade dysplasia and three with atypical squamous cells of uncertain significance (ASCUS). Table 5.1 shows the cytology result by brushing site. The risk of an abnormal cytology result in brushings taken from an abnormal appearing site was 4.0 (95% CI: 1.5-10.3) times greater than the risk of an abnormal cytology result in brushings taken from a normal appearing brushing site.

Of the cases with high grade or malignant cytology, 44% (8/18) of cases had additional grades of dysplasia present. One malignant case also had cells with high grade dysplasia present, four malignant cases showed both low and high grade dysplasia, and three samples with high grade dysplasia had cells with low grade dysplasia. Images of low grade, high grade, and malignant cytology results are shown in Figure 5.3. All eight of the cases with a continuum of dysplasia were seen in p16 positive cases.

Table 5.1: Cytology results by tumour or non-tumour site.

		Cytology result				Totals
		Negative	Low grade	High grade	Malignant	
Brushing site	Tumour	14	3	11	6	34
	Non-tumour	31	5	1	0	37
Totals		44	8	12	6	71

5.4.6 Results by tumour site

When looking at brushings of the tumour site from p16 positive cases, 100% (3/3) from patients under anaesthetic were positive for HPV 16 DNA. Of the tumour site brushings from conscious patients, 63% (17/27) were positive for HPV 16 DNA. Further analysis was not possible due to the low numbers of brushings taken from patients under anaesthetic. Of the remaining ten HPV 16 DNA negative samples; four were base of tongue samples, two had recently had the affected tonsil removed and two had abnormal cytology.

There were 24 cases where brushings were taken from a tonsillar tumour site. Of these, HPV 16 DNA was detectable in 75% (18/24). There were nine brushings from a base of tongue tumour site in which HPV 16 DNA was detectable in 44% (4/9).

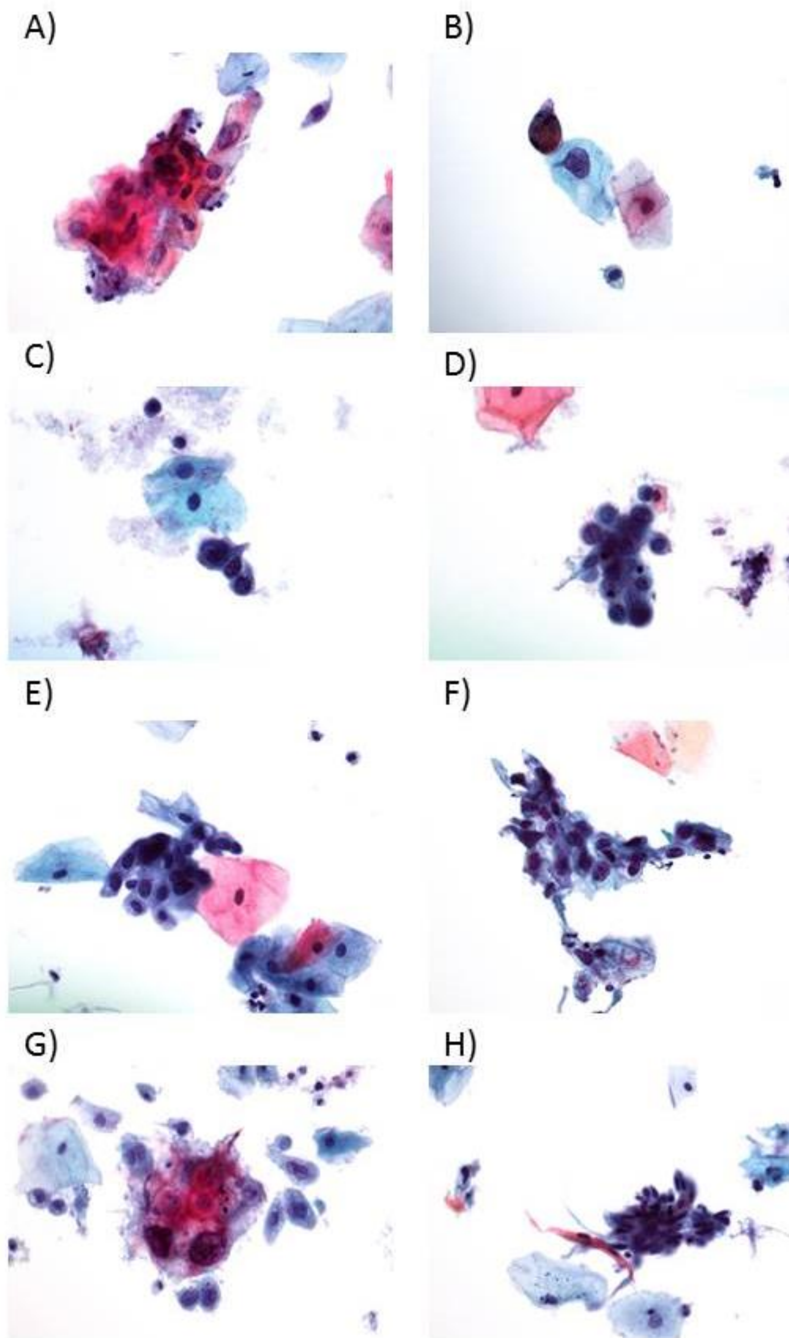

Figure 5.3: Images of abnormal cytology. Magnification 40x, ThinPrep® Papanicolaou Stain. A & B) Low grade changes. Low grade cells show nuclear enlargement and hyperchromasia relative to surrounding normal cells. C - F) High grade changes. High grade cells are small parabasal sized cells with nuclear hyperchromasia and irregular nuclear membranes. G & H) Invasive carcinoma. Cells are pleomorphic with marked nuclear atypia with tumour diathesis in the background.

5.5 Discussion

The detection of HPV 16 DNA in tonsillar brushings from known oropharyngeal cancer patients seen in this study is consistent with other published studies [125,167]. HPV DNA has previously been detected in the tonsils from cancer cases and cancer free individuals by either brushings taken under anaesthetic [123,125] or the use of ex vivo brushings [126,127]. Approaches using patients under anaesthetic limit the interpretation of how brushings could be applied as a screening test as the procedure imparts significant financial and time costs, and risks for the patient. Our results show in brushings from conscious oropharyngeal cancer patients HPV 16 detection appears to be superior for tumours of the tonsillar sub-site over base of tongue carcinomas with 18/24 and 4/9 respectively having detectable HPV 16 DNA. The base of tongue is defined as “the posterior attached portion of the tongue extending from the line of the circumvallate papillae to the junction of the base of the epiglottis”[169]. However, it is possible that samples that were taken from the base of tongue from conscious patients may have been posterior oral tongue specimens as access to the base of tongue is often prevented by the pharyngeal reflex [126]. Interestingly, half of the base of tongue samples were positive for HPV 16 DNA, and had abnormal cytology compared to all four HPV 16 negative base of tongue samples having normal cytology. This suggests the periphery of the tumour may have been able to be sampled in half the cases even though on visual examination there was no abnormality seen.

This study detected HPV 16 DNA in 83% (10/12) of samples taken from p16 positive cases with visible tumours. Of the remaining two samples one case had high grade cytology and therefore may be attributed to a non-HPV 16 type. The final brushing was recorded as taken from the right tonsil and the appearance recorded as visible tumour.

However, the pathology reports state the tumour was on the left tonsil. It is therefore uncertain if the tumour was actually sampled. Regardless of this sample, these results from visible tumours and the overall 21/30 (70%) of HPV 16 positive brushings from p16 positive samples are comparable to the 28/32 (88%) of p16 positive oropharyngeal cancer samples with high risk HPV in brushings taken under anaesthetic during parendoscopy seen by Kofler *et al* [125]. Although the number of cases in our study was small, the results show HPV 16 DNA can frequently be detected in superficial brushings from the tonsils of conscious p16 positive OPC patients.

This study had one p16 negative case that had detectable HPV 16 in the brushing sample. This case also had low grade changes on cytology, however adequate RNA was not able to be extracted. This patient was an 87 year old woman with a metastatic poorly differentiated carcinoma. When reviewing the pathology report, it was noted that the p16 immunohistochemistry was performed on a cell block from a fine needle aspiration sample. There are no set guidelines for what constitutes a positive p16 result in a cell block specimen, and if the cut off of 70% of tumours cells stained in the histology specimens is applied, there is likely to be a high rate of false negatives [170]. It is also possible the HPV detected was simply a transient infection and unrelated to the tumour [47,136].

HPV 16 mRNA was detectable in 55% of HPV 16 DNA positive samples after excluding 25% of samples due to a lack of amplifiable mRNA. To the best of our knowledge we are the first to apply RNA based tests to brushings for oropharyngeal cancer. The advantage of an RNA over a DNA based test is a higher specificity for disease due to the detection of transcriptionally active HPV [93]. However, results from this study

showed the detection of HPV 16 DNA correlated well with both p16 results and the brushing site. HPV 16 DNA detection was rare in samples taken from a non-tumour site. Overall HPV 16 DNA detection was superior to cytology. This scenario is also seen in cervical screening which is likely to result in a move to primary HPV testing in the near future [121,171].

In this study, 1mL of sample for was used for RNA extractions. However, the high number of beta-globin negative samples suggests using more sample volume may be required. It should also be noted that the gross appearance of brushings varied considerably from transparent to very turbid or heavily blood-stained. This likely occurred from a combination of whether the tumour or a normal tonsil was sampled and the patient's ability to tolerate the procedure. It is also possible that factors such as patient age, time since last meal or drink, and oral health may have played a role. A limitation of our study was we had multiple people collecting the samples. Although all were trained on how to do the procedure, it may have had an impact on the quality of the specimens collected.

This study describes cytological changes comparable to cervical pre-cancerous lesions. A continuum of cytological changes, ranging from low to high grade dysplasia, high grade dysplasia to invasive carcinoma, or low grade dysplasia to invasive carcinoma was seen in eight of the 18 samples with abnormal cytology. The continuum of changes was seen only in p16 positive cases, and may suggest progression similar to that seen in cervical cancer. None of the low grade changes observed in this study included koilocytes. This is consistent with finding by Francheschi *et al* who found only one koilocyte in a study of 200 tonsillectomy samples removed due to benign diseases

[127]. Koilocytes are a common finding in low grade cervical neoplasia, and are a result of productive HPV infection and the production of virions in a mature squamous cell [5]. Koilocytes are not generally associated with malignancy as they are found in productive infection. By comparison, in cervical screening it is common to see non-koilocytic low grade changes in a smear with a high grade lesion. To date, studies of tonsillar specimens from cancer free individuals have not found any evidence of pre-cancerous lesions for OPC [123,127,166]. However, areas of dysplasia and carcinoma in situ associated with oropharyngeal cancer have been described [81]. This may support the idea that although HPV infection in the tonsil is rare, the proportion of lesions that progress may be higher than in the cervix [166]. Further research into the histological occurrence of and appearance of dysplastic changes in HPV positive OPC is required, and should be conducted on those with OPC. The natural history of HPV in the tonsil remains uncertain and describing precancerous lesions adjacent to an existing tumour is more feasible than detecting pre-cancers in studies of the general population. Until the lesions seen in this study are well described histologically, their significance cannot be ascertained.

The absence of well-defined pre-cancerous lesions is a major issue when looking at the potential for screening for oropharyngeal cancer. Fakhry *et al* found associations between HPV 16 detection and abnormal cytology in those with visible oropharyngeal lesions. They then attempted to see if similar associations were present using a nested case-control study of 401 human immunodeficiency virus (HIV) infected individuals [123], a group known to be at higher risk HPV positive oropharyngeal cancer [155]. This case-control study found no association between the presence of HPV 16 DNA and the presence of cytological abnormality. They attribute this to not being able to

adequately sample the site of infection and therefore cytological abnormality in the tonsils [123]. Similar criticisms of any brush based pap-test equivalent have been described by several authors [124,127,165]. Although HIV infected individuals have a higher risk for oropharyngeal cancer, other factors need consideration. For example, the study population used by Fakhry *et al* consisted of 60% males and almost 90% African Americans, and had a median age of 46 years [123]. By comparison, a study of 271 oropharyngeal cancer cases from 1984-2004 in the United States with an overall HPV prevalence of 44%, found 80% of patients were male, 63% were European and 80% of cases were aged over 50 [46]. Therefore, the Fakhry *et al* study population may not have been adequately designed for the early detection of OPC.

It is currently unknown if the early detection of HPV positive OPC would decrease the mortality from the disease [108]. A focus on the early detection of OPC to minimise the significant morbidities from current treatment regimens may prove more useful than the quest for precancerous lesions. Treatment for OPC often involves high dose radiation and side effects of treatment have significant impact on patient quality of life [53]. The detection of micro-lesions confined to the tonsil by narrow band imaging has been previously described and although the follow-up time is limited the treatment was a surgical approach with no requirement for radiation [172,173].

Overall, this study is the first to use brushings from conscious patients to detect HPV 16 DNA, RNA, and cytological abnormalities which has shown to be a simple procedure that is acceptable to patients. We have described cytological abnormalities comparable to cervical precancerous lesions and shown a continuum of dysplasia in a

number of p16 positive cases. Further research and histological confirmation of these apparent precancerous lesions is required before their significance can be determined.

Chapter 6 - General Discussion

6.1 Introduction

This thesis has assessed the burden of HPV positive oropharyngeal cancer (OPC) in New Zealand, and investigated diagnostic methods and possibilities for early detection in a pre-vaccine population. We have determined the prevalence and genotypes associated with HPV positive OPC in New Zealand; compared a range of tests for the detection of HPV in archived formalin fixed paraffin embedded (FFPE) oropharyngeal cancer cases; described the demographics and exposures of a pre-vaccine population of oropharyngeal cancer patients and used tonsillar brushings from conscious patients to investigate the detection of HPV 16 and cytological abnormalities. The following discussion puts these findings into context, discusses their significance and suggests areas for further research.

6.2 Summary of results

In the study population, the incidence of oropharyngeal cancer increased between 1996 and 2012, and the proportion HPV positive increased from 61.9% (95% CI: 40.9% – 79.2%) in 1996-98 to 87.5% (95% CI: 79.8% – 92.5%) in 2010-12. Almost all (98.5%) HPV positive cases were positive for HPV 16, with one case each of HPV 33 and 35. There was a statistically significant association between HPV positivity and date of diagnosis (OR: 5.65, 95% CI: 2.60-12.30, 2010-12 vs 1996-98), and between HPV positivity and patient age (OR: 0.55, 95% CI: 0.33-0.99, ≥61 years vs ≤60 years). Further work on risk factors for HPV positive OPC (Chapter 4) found a significant association between a p16 positive tumour and having ever given oral sex (Adjusted OR: 15.9, 95% CI: 1.4-230.5). These OPC patients (regardless of p16 status) had significantly increased

exposures to asbestos and formalin than participants in the New Zealand working survey.

The finding of focally positive p16 staining and discrepant p16/PCR results in Chapter two led to further investigation on the causative role of HPV in cases where the true HPV status was uncertain. When comparing p16 to the gold standard of E6/E7 mRNA detection, Chapter three identified ten cases with an incorrect HPV status based on the use of p16 alone. These results highlight the need for better diagnostic tests, especially if treatment decisions will be based on tumour HPV status.

The investigation of brushings from conscious OPC patients (Chapter 5) found the detection of HPV 16 DNA was more common when samples were taken from an abnormally appearing site. Cellular changes comparable to cervical precancerous lesions were present in 27 of the 32 samples with abnormal cytology, and of these 27 samples, the eight that showed a continuum of dysplastic change were all p16 positive.

6.3 Current difficulties in the determination of HPV status in oropharyngeal cancer

Although there is a call for the development of clear guidelines for the interpretation of p16 in OPC akin to the LAST guidelines for anogenital specimens, it is questionable that these guidelines could be developed. This is because there is no consistent cut off point or set of staining characteristics that provides certainty in identifying transcriptionally active HPV. This fact was shown in results from Chapter three, and also in Chapter two where all focally positive p16 cases and cases with discrepant p16/PCR results were excluded from analysis as their HPV status was uncertain. In the absence of clear guidelines, it is likely that when seen in the diagnostic setting, focally

positive cases will be reported as negative, because they do not meet the commonly used arbitrary threshold of 70% of tumour cells stained [44,47,62,88,89]. Another possibility is they are reported as focally positive (this was seen in diagnostic pathology reports provided with specimens for Chapters two and five). This reporting of intermediary p16 results may lead to confusion in the clinical interpretation of HPV status, and therefore the prognostic information given to patients.

The major concern with the inability of p16 to reliably determine the HPV status of oropharyngeal tumours is the current interest in the use of de-intensified therapies for HPV positive cases [53]. Results from Chapter three identified six patients that would have been wrongly classified as HPV positive, and a further four as HPV negative if p16 alone was used. Assuming treatment decisions were based on p16 results, these six false positives would receive lower doses of chemo- or radio- therapy which could impact on their cure rate and long term survival [53]. Moreover, the four HPV false negatives would receive unnecessary high intensity chemo- or radio- therapy with significant risks for long term morbidities and impacts on quality of life [53,56,174].

Although the inappropriate treatments for these patients are hypothetical at this point, the impacts of high intensity therapies are very real, and even more significant in OPC due to the younger age of the patients affected [53]. In the USA, there has been a significant increase in the number of OPC patients aged under 45 [175]. This means at the time of diagnosis many individuals may have full time employment, and dependent families. Post treatment, OPC survivors have decades of life to live [174]. Long term morbidities for head and neck cancer patients are numerous and the most significant are problems with speech and swallowing [54-56]. Patient quality of life is

further impacted by the flow on of such affects leading to withdrawing from social situations and not maintaining nutritional status [174]. Those who have had radical neck dissections as part of their treatment also potentially face self-esteem and appearance issues due to facial disfigurement [55]. In addition, shoulder dysfunction is common post neck dissection, due to damage to accessory nerves, which can impact on the patients return to work, and ability to carry out routine tasks [174]. Not surprisingly, the rates of depression, and subsequently suicide are high in head and neck cancer survivors [176,177]. The risks of these morbidities highlight that the key factor that needs to be addressed before treatment de-escalation is the reliable determination of tumour HPV status for every patient.

The current gold standard for the determination of the HPV status of an oropharyngeal tumour is the detection of HPV E6/E7 mRNA [86]. This method reliably establishes causation, as it detects transcriptionally active virus [47,86]. Although mRNA can be detected in FFPE blocks, the usual sample required is fresh tissue [86]. This adds a further layer of complexity as the usual specimen type in diagnostic histopathology laboratories is FFPE blocks. In diagnostic laboratories in New Zealand PCR based tests (such as those for HPV) are frequently carried out in specialised molecular departments often present only in major centres. Because of this the ideal test for OPC HPV status would use FFPE samples and be able to be run on existing platforms seen in the majority of laboratories.

The detection of HPV E7 by IHC would provide an ideal marker for HPV status in OPC. Not only would it be specific for HPV, it would detect transcriptionally active HPV, and be accessible and applicable for the workflow in many laboratories. Despite numerous

attempts to optimise such an assay, we were unsuccessful to do so. We trialled three commercially available E7 antibodies and an important challenge we encountered was non-specific staining in many tissue types. If not for the financial and time constraints of the PhD, further work on such an assay would have continued. Ramirez *et al* developed an in-house HPV 16 E7 antibody, the assay detected HPV E7 in 100% (22/22) of CIN3 and invasive cervical cancers [178]. In addition, E7 presence in low grade lesions correlated with progression to high grade lesions [178]. However, they also acknowledge their E7 antibody has some reactivity with the stroma, which is evident in the published images [178]. It appears E7 may be a particularly problematic target: it is expressed at low levels [179], it has a short half-life [16,179], there are several conformations, including a dimer and oligomer, which are more common in transformed cells [178], and much is still unknown about possible post-translational modifications and processed or intermediate forms of E7 present in transformed cells [179]. Regardless of these challenges, the detection of HPV E7 and other transcriptionally active viral proteins, such as E6 [34], by IHC, remains a viable option for a sensitive and specific assay to determine tumour HPV status. The development of such detection methods warrants further research.

6.4 The role of vaccination in prevention of oropharyngeal cancer

The cervical cancer vaccine (Gardasil®) is the best option for the primary prevention of HPV positive OPC in the future [108,165]. The vaccine has been shown to be effective against cervical infection and precancerous lesions, genital warts, and anal precancerous lesions [32,108,117,119]. The evidence of its effectiveness in non-cervical sites [119] indicates it will likely prevent infection in the oropharynx. There are currently no studies that have been specifically designed to determine vaccine efficacy

in oropharyngeal cancer. However, Herrero *et al* calculated a vaccine efficacy of 93.3% based on the detection of HPV in oral rinses four years post vaccination [120]. In the Herrero study there was one oral HPV infection in the vaccinated group, however as no oral rinses were taken at baseline this may have been present prior to vaccination, if so the vaccine efficacy would be higher than that reported. Similarly, Gillison *et al* in a study of 2275 woman found an oral HPV prevalence of 0.5% in the unvaccinated cohort, and 0% in the vaccinated cohort [82].

Even in the unlikely scenario that the vaccine cannot prevent oropharyngeal infection, it will still have a significant impact on the incidence of OPC as it will reduce the reservoir of genital HPV and thereby prevent transmission to the oropharynx [108]. This prevention of transmission to the oropharynx will require adequate vaccine coverage that in many developed countries is a major barrier due to controversy surrounding the vaccine. The controversy has been attributed to the fact that HPV is a sexually transmitted infection, and concerns over vaccine safety [32]. The latter has had significant impacts including the Japanese government suspending recommendations for the vaccine three years after it was introduced [180].

The anti-vaccination (anti-vax) movement appears more prominent in recent times largely due to the availability of information (regardless of scientific evidence) on the internet and through social media. However, public knowledge of HPV and its role in cancer is limited [181,182]. After personal communication with 20/54 study participants during recruitment many had only “found out” about HPV after their diagnosis, and some were still unaware of the role of HPV in cancer post-diagnosis, when recruited. Many current patients have children of an age to be eligible for the

vaccine, and this group have seen first-hand the effects of HPV related cancer. It is possible the improved understanding of HPV in this group could help with increased vaccine coverage, in a local/community setting.

The vaccine currently used in New Zealand (Gardasil® 9) contains HPV types 16, 18, 6, 11, 31, 33, 45, 52, and 58 [109]. HPV type 35 is not included. Both our research and the study by Ou *et al* [130] found one case of HPV 35 positive OPC in New Zealand populations. Interestingly, neither the Ou *et al* study nor our research found any HPV 52. HPV 52 is a high risk HPV type found in over 20% of histologically confirmed high grade (CIN3) cervical biopsies in New Zealand [183]. This is significantly higher than the reported 2%, 5% and 14% of HPV 52 positive CIN3 cases in Europe, Northern America and Australia respectively [183]. An early hypothesis in this study was that there would be a higher proportion of HPV 52 positive OPC cases than reported in international studies. This was based on the reasoning that New Zealand has a higher proportion of HPV 52 in the circulating pool of HPV, and therefore there is high potential for its spread to the oropharynx. The absence of any HPV 52 suggests that although it is a high risk type, it may have a lower oncogenic potential than HPV 16, or there may be differences in tissue preferences between viral types. The prevalence of HPV types shown in New Zealand OPC provides further support for the future prevention of OPC through vaccination.

Some degree of herd immunity from Gardasil® vaccination has been described when over 50% of a population are vaccinated [115]. New Zealand currently has a coverage rate of 67% for the 2003 birth cohort [113], suggesting population effects will be seen in the future. Claims have recently been made in Australia that based on the timing of

vaccine introduction and coverage rates seen to date, they would be the first country to eliminate cervical cancer [184]. The vaccine was introduced in New Zealand at a similar time to Australia and we have comparable coverage rates, suggesting the elimination of cervical cancer is also possible here. This poses some questions around resource allocation in developed countries where incident OPC and cervical cancer case numbers are approximately equal, and the post treatment morbidities seen in OPC survivors have significant long term personal and economic consequences.

It is expected in 2021, cervical screening in New Zealand will switch from cytology to primary HPV screening (using a PCR test). There will be costs associated with this change including increased administration and support services, more colposcopy referrals for unvaccinated women, and an increased cost per test from $31 for cytology to $35 for an HPV test. The increase in test cost will largely be offset by increasing the screening interval from three to five years [185]. The necessity of this change to primary HPV testing however, should be questioned. In New Zealand, the incidence of cervical cancer has remained fairly steady for the last two decades, with between 141 and 180 cases per year for 2005-2015 [186] with the majority of cases seen in non-screened or under-screened women. Currently only three of the 20 District Health Boards (DHB) meet the coverage target of 80% of eligible women having had a smear in the past three years, and coverage has reduced in 15 DHBs [187].

Those vaccinated during the catch-up program (offering vaccination to those born during/after 1990) and those vaccinated up to 2010 may now be enrolled in the national cervical screening program. Based on the use of cytology the current cost of

the screening program for unvaccinated women is $31.7 million per year [185]. This cost includes initial testing, follow-up appointments, histology, and colposcopy services. Models for cost effectiveness assuming a vaccine coverage rate of 54% have shown this cost to be $25.9 million for a vaccinated cohort [185], a saving of $5.8 million per year. This cost reduction will be greater with improved vaccine coverage, and once those vaccinated with Gardasil®9 are of screening age, as the modelling was based on the use of Gardasil®4. Furthermore the estimated eradication of cervical cancer by around 2040 [184], suggests cervical screening may only be required in the short term.

On top of the costs of screening, each case of cervical cancer costs the health system $23,116 and other HPV related cancers likely bear similar costs. Investment in vaccination represents the most cost effective strategy long term for the prevention of all HPV associated cancers.

6.5 Possibilities for the early detection of HPV positive oropharyngeal cancer

Although vaccination will likely lead to dramatic reductions in OPC in the future, this will take decades as those who are vaccinated must be in in at least their forties before effects are seen.

Once we had established the burden of HPV positive OPC in New Zealand, we began to explore what might be possible in terms of early detection. The first step was to establish if a minimally invasive pap-test equivalent taken from conscious individuals was capable of detecting HPV (Chapter 5). To date many studies have used anaesthetised patients [123,125] or ex vivo brushings [126,127] which are useful for

the simple detection of virus, but limits test practicalities. Furthermore, we sought to investigate any precancerous lesions that may occur adjacent to the tumour using cytology. Precancerous lesions for OPC are yet to be described, but adjacent dysplasia is described in the literature [81], and in 33% of pathology reports from Chapter two research. p16 staining in the adjacent epithelium of cases from Chapter two was also recorded.

Both the PAP2 and SPLIT studies aimed to detect precancerous lesions for HPV positive OPC in healthy individuals, and failed to find any such lesions [123,126]. As discussed in Chapter five, the study population for PAP2 was not optimally designed for OPC, and similar concerns can be raised with the SPLIT population. The SPLIT study population consisted of 200 patients aged over 15 years with a mean age of 30.3 years, of which 137 participants had undergone tonsillectomy for infectious reasons [127]. By comparison, our study was of known oropharyngeal cancer patients. From my work in cervical screening I was used to seeing the continuum of cytological changes present in cervical cancer samples and thought the same continuum may be seen in HPV positive OPC. The use of oropharyngeal cancer patients to explore lesions adjacent to known HPV positive tumours is a useful approach when so little is known about the natural history and progression of this disease. It is uncertain how long before the development of cancer such lesions will be present, and based on the study population demographics of the PAP and SPLIT studies they have assumed there will be a long lag time of 10-20 years as seen in cervical cancer.

The cytological abnormalities we have identified are comparable to cervical precancerous lesions. Cellular changes reported in Chapter five were coded using the

Bethesda reporting system, and applied cervical criteria for low / high grade and malignant changes. The applicability of this system highlights similarities in the appearance between these changes and cervical lesions. Interestingly the continuum of low / high grade to malignant changes seen within samples was only present in p16 positive cases. Based on cytology alone these observed changes cannot be classified as precancerous lesions for OPC. Histological confirmation of the location of these cells in relation to the tumour, and molecular studies of the presence of transcriptionally active HPV within these cells is required. HPV behaves similarly in all anogenital cancers with precancerous lesions for cervical, anal, penile, vaginal and vulvar cancers being well described [14]. The changes observed in this study suggest similar behaviour of HPV in OPC.

Because of the low incidence of OPC, difficulties in sampling the tonsillar crypts, and the current lack of any defined precancerous lesions, population based screening as is used for cervical cancer is not a feasible option [128,165]. However, the pursuit of early detection of smaller tumours, ideally without regional metastases, remains a worthy one. Currently, the majority of OPC are detected when there is involvement of regional lymph nodes [46]. In theory, the detection of smaller localised tumours would result in reduced need for chemo- or radio- therapy and permit more surgical therapy being used. One such approach is trans oral robotic surgery (TORS). This is a new technique allowing tumour resection through the open mouth instead of by open surgery [53]. Early data on the use of TORS shows improved functional and oncologic outcomes for OPC patients [188].

Chapter five reported that brushings taken from conscious patients can reliably detect HPV 16 DNA when there is an oropharyngeal abnormality. These recorded brushing site abnormalities ranged from slightly enlarged or irregular, to visible tumours and occurred in 50% of participants with recorded site appearances. The use of brushings from conscious individuals is important as it could in the future possibly translate to a point of care test that may provide referrals from primary care to Ear Nose and Throat (ENT) services. Currently, ENT referrals are at the GPs discretion and may vary between GPs and practices. From my first hand collection of samples, the brushing procedure was well tolerated by patients with only minimal discomfort. Only one participant I sampled refused to have an adjacent site sample once the first brushing was taken and this was not due to the procedure, but instead the pain associated with holding his jaw open due to the location of the tumour.

Aside from a mass in the neck the symptoms of OPC can be vague. Consistent with other published data [189], Chapter four reported that many patients experienced a sore throat, pain when swallowing, earache, a feeling of something being stuck in the throat, and hoarseness of the voice in the six months before their diagnosis. These symptoms are often associated with more benign conditions, and taking brushings from every patient with a sore throat would not be a feasible early detection approach for HPV positive OPC. The development of a set of criteria, or a simple questionnaire to determine risk and thus prioritize who has brushings taken should be considered. Based on finding from this thesis, criteria could include: appearance of the tonsils, the presence and progression of symptoms, and a brief questionnaire on sexual behaviours and exposures to hazardous substances.

Part of this further research on the use of brushings for early detection would need to carefully consider what would happen after the detection of HPV 16 in a sample. The simple detection of virus may not be associated with the presence of cancer, however, study participants in this research have reported neck masses have appeared over a matter of days, and others that the neck masses over doubled in size in two months. It has been suggested HPV infection in the oropharynx is more likely than HPV infection in the cervix to progress to cancer [166], and OPC is also more likely to metastasize regionally than cervical cancer [190]. The aggressive behaviour of these tumours suggests that any follow up after the detection of HPV 16 may need to be rapid. Hypothetically, performing a tonsillectomy on all those with HPV 16 positive brushings would likely help in the description of any precancerous lesions, and could in itself be a curative treatment. However, tonsillectomy is not without its own risks and involves considerable use of finite healthcare resources. Other approaches could include the use of narrow band imaging technology which has in limited case reports been used in the detection of very small OPC [172,173].

The thematic analysis of free text comments also revealed four reports of a delay between presentation of symptoms to primary health care and final diagnosis. These comments fell below the required number (six) to be identified as a theme but nevertheless represent an important issue in OPC. These participants' accounts mirror a recently published case study [191]. In the case study, a woman presented to her primary care physician with left ear pain and received antibiotics and increasing doses of pain medication over the next 23 months, until she had speech difficulties and was referred for an MRI. This confirmed a large mass which was an HPV positive OPC [191]. In the primary care setting, there needs to be awareness of OPC as a potential

diagnosis, particularly in younger, otherwise healthy patients. Recently, Whanganui District Health Board organised an inter-professional education evening raising awareness on HPV positive OPC targeted at those working in primary healthcare. Approaches such as this are important as it is possible an earlier referral to ENT services could lead to earlier diagnosis.

6.6 Risk factors for oropharyngeal cancer

The descriptive analysis presented in Chapter four was initially designed to investigate risk factors for HPV positive OPC. However, due to the high prevalence of HPV positive OPC in our sample size of the comparison group (p16 negative cases) was very small (n=8). This small comparison group limited the analysis to some aspects of the questionnaire. The final multivariable model did however find ever having given oral sex to be associated with having an HPV positive tumour, showing some risk factors for HPV positive versus negative OPC could be assessed, and this finding was consistent with international data [63,64].

Changes in sexual behaviours are thought to be the likely cause of the increase in HPV positive OPC seen in developed countries. The increase in cases began in the 1970s which is around the time oral sex was said to be popularised or considered mainstream [192]. Sexual behaviours have continued to change and, with the exception of the late 1980s/early 1990s due to fear around the emergence of HIV/AIDS, high risk behaviours have likely increased [192]. The last cohort of unvaccinated individuals (those born in the late 1980s) were among the first to have widespread access to mobile phones, leading to the emergence of sexting (the sending of sexually explicit text messages), which is linked to sexual risk taking in young adults [193]. Furthermore, during the

same time, advances in technology have led to the development of apps such as Tinder, which could be seen to encourage casual sex. This cohort may carry a higher risk than previous populations for the development of HPV positive OPC, suggesting the incidence of OPC in developed countries will continue to rise in the short term.

International literature states there is an approximate 4:1 ratio of males to females for OPC [43,50]. A study of 382 OPC cases from 2002-14 in Poland found 32% of p16 positive cases were female, compared to 18% of p16 negative cases [194]. A study comparing the incidence of oral cancer (predominantly smoking/alcohol related) and OPC (predominantly HPV related) in New Zealand and Queensland, Australia, found a statistically significant increase in the number of female OPC cases of 2.1% per year from 1982, but no change in oral cancer incidence in females [131]. It was reported in Chapter two that when using the p16/PCR algorithm there was no HPV positive OPC in a female in 1996-98. Using the same algorithm, in 2010-12 20% (18/91) of OPC cases were in females. However, caution should be applied in the interpretation of these results due to the small number of cases, particularly in the 1996-98 period (n=21).

Reasons for the apparent subtle changes in the gender distribution of HPV positive OPC are likely to be complex, and although subtle they warrant discussion due to a number of social changes that have occurred since HPV positive OPC was first described.

If we assume that giving oral sex was as likely to be done by men as women [192], then why is HPV positive OPC a male dominated disease? There may possibly be some degree of protection for oral infection seen in women who have had previous genital HPV infection [82]. The age at first oral sex has decreased over time and may now

occur prior to first vaginal sex [192]. This change may have reduced the protection for women described above, as the first HPV infection exposure may now be oral.

Another factor may be that the viral load is higher in the vagina than penis, therefore explaining the higher rates of HPV positive OPC in men [36]. An increase in the incidence of HPV positive OPC in females may in part be contributed to an increase in the number of same-sex encounters in females over the past few decades [195].

Declining rates of male circumcision in developed countries [196] may also have an impact on the incidence of HPV positive OPC in females. Circumcision results in lower rates of HPV infection, higher clearance, and lower viral load infections in the penis [197].

When looking at other risk factors for OPC, comparisons of the exposures of OPC patients to the New Zealand working survey in Chapter four found OPC patients (regardless of p16 status) were significantly more exposed to formalin and asbestos than those that participated in the New Zealand working survey. As discussed in Chapter four, there is some existing evidence for a role for these substances in head and neck cancers and both are classified as human carcinogens by IARC [152,154]. It is important to note that our findings are based on all 89 oropharyngeal cancers, and was not limited to only p16 positive cases. It is uncertain if these substances may interact in some way with HPV. For example, could the inhalation of these substances lead to irritation of the mucosa and therefore facilitate the entry of HPV? It could also be possible that these substances promote the transforming actions of HPV. The plausibility of a role of co-factors that cause irritation of the mucosa is supported by cofactors seen in cervical cancer. The presence of *Chlamydia trachomatis* is a widely

accepted cofactor for the development of cervical cancer [6]. The presence of *C. trachomatis* creates chronic inflammation in the cervix impacting on viral persistence and progression [198]. Further work on the role of inhaled hazardous substances in HPV positive is required.

6.7 Conclusions

This research has shown HPV positive OPC is increasing in incidence and represents a significant burden on the New Zealand health system. Although there are similarities between cervical cancer and HPV positive OPC the knowledge of one cannot simply be applied to the other. This is clearly shown by the lack of clear guidelines around p16 testing and the current misclassification of tumour HPV status in OPC, and the fact that population based screening as seen for cervical cancer is not feasible for OPC. In New Zealand, based on having 120 cases per year for the next 30 years, there will likely be around 3000 new HPV positive OPC cases until the effects of vaccination are seen. It is currently unlikely we can prevent these cases. Future research into the development of early detection methods to limit morbidities and improve post-treatment quality of life is the best hope for these patients.

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RESEARCH ARTICLE

The prevalence of human papillomavirus in oropharyngeal cancer in a New Zealand population

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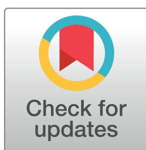

OPEN ACCESS

Citation: Lucas-Roxburgh R, Benschop J, Lockett B, van den Heever U, Williams R, Howe L (2017) The prevalence of human papillomavirus in oropharyngeal cancer in a New Zealand population. PLoS ONE 12(10): e0186424. https://doi.org/10.1371/journal.pone.0186424

Editor: Maria Lina Tornesello, Fondazione IRCCS Istituto Nazionale dei Tumori, ITALY

Received: June 13, 2017

Accepted: September 29, 2017

Published: October 19, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The funder (MedLab Central Ltd) provided support in the form of salaries for authors [BL, UV, RW], but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Abstract

Background

The incidence of oropharyngeal cancer (OPC) in New Zealand (NZ) has more than doubled over the last 14 years with 126 cases in 2010. Overseas studies have shown that human papillomavirus (HPV) plays a significant role in the development of these cancers. However, the role of HPV in OPC and the burden on the NZ health system is unclear.

Aim

The aim of the study was to determine the prevalence and the genotypes of HPV associated with OPC in New Zealand.

Methods

In this study, 621 OPC were identified from cancer registry data from 1996–98, 2003–05, and 2010–12. Biopsies of 267 cases were then retrieved from laboratories throughout New Zealand. p16 immunohistochemistry and a human beta globin PCR were performed on all specimens. HPV genotyping was performed on all beta globin positive specimens using real-time PCR with melt analysis.

Results

Using a p16/PCR algorithm, 77.9% (95% CI: 71.1–83.5%) of cases were attributable to HPV. Of these, 98.5% were HPV 16 positive. There was also one case each of HPV 33 and 35. The percentage of HPV positive cases increased from 61.9% (95% CI: 40.9%– 79.2%) in 1996–98 to 87.5% (95% CI: 79.8%– 92.5%) in 2010–12. Results from the multivariable model, adjusted for sex and ethnicity found statistically significant associations between HPV positivity and timeframe (OR: 5.65, 95% CI: 2.60–12.30, 2010–12 vs 1996–98), and

Competing interests: The authors have read the journal's policy and have the following competing interests: Authors (BL, UV, and RW) are employed by MedLab Central Ltd. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

between HPV positivity and patient age (OR: 0.55, 95% CI: 0.33–0.99, ≥61 years vs ≤60 years).

Conclusions

This data is consistent with data from other developed countries showing an increase in cases of HPV positive OPC in New Zealand, and the majority of cases being attributable to HPV 16. These results support the recent inclusion of males into the nationally funded immunization schedule for Gardasil® 9.

Introduction

Head and neck squamous cell carcinomas (HNSCC) comprise cancers of the oral cavity, larynx, hypopharynx, and oropharynx [1]. The incidence of HNSCC in developed countries has decreased in parallel with a decrease in the number of individuals that smoke [2,3]. However, the incidence of oropharyngeal cancers (OPC) has increased [4]. The proportion of cases reported to be due to human papillomavirus (HPV) varies considerably between studies and the variation is dependent on the population studied, location of the tumour, and method of HPV detection [5]. HPV 16 is a recognised carcinogen in the oropharynx, while there is limited evidence for the role of HPV 18 in OPC, and insufficient evidence for other HPV types [6]. Worldwide over 90% of HPV-positive OPC are due to high risk HPV 16 [7], with as many as 97% of cases reported by some studies to be attributable to HPV 16 [8,9]. Other high risk HPV types such as types 18, 31, and 33 have also been detected [7,9,10].

In the past, the majority of HNSCC was seen in the larynx and oral cavity and was associated with smoking and heavy alcohol consumption [4]. The increasing incidence of HPV-positive cancer of the oropharynx (including tonsillar and base of tongue cancers) over the last three decades is thought to be due to changing sexual behaviours and this is represented in the risk factors for the disease [4,11,12]. Data from the US, Australia, and Sweden has shown an increase in risk factors such as the occurrence of premarital sex, oral sex, the number of lifetime partners, and a reduction in the age of sexual debut in recent birth cohorts [13]. Other risk factors include marijuana use and diet and nutrition [14,15].

The number of cases of OPC caused by HPV in New Zealand is unknown, however, when looking at anatomical site alone there has been an approximately threefold increase in the number of OPC cases from 35 cases in 1996 to 126 cases in 2010 (Ministry of Health, 2014). In contrast, the incidence of oral cavity cancers has remained steady during the same time period [16]. Currently there is no published data on the contributions of various HPV types to OPC in New Zealand. It is likely New Zealand's cases follow international data with HPV 16 responsible for the vast majority of cases. Thus, this study aims to determine the prevalence and the genotypes of HPV associated with OPC in New Zealand.

Materials and methods

Ethics approval was obtained from the Health and Disabilities Ethics Committee (reference: 14/STH/128). Participant privacy was preserved by de-identification of samples.

Participant recruitment and sample collection

Cases were identified by a New Zealand cancer registry search of all cases of oropharyngeal cancer of ICD-10 codes C01, C05.1–5.2, C09.0–9.9, and C10.0–10.9 from 1996–98, 2003–05,

and 2010–12. The New Zealand cancer registry is a population based register of all primary malignant diseases diagnosed in New Zealand. The information available from the registry comprises: patient demographics (age, sex, and ethnicity) and tumour specific information (tumour site, morphology, grade, extent, and diagnosis date, laboratory code and basis for diagnosis (histology, cytology etc)). Data was cross checked with National Health Index (NHI) data to identify patients that were deceased. Study information sheets and consent forms were sent to all living patients whose samples were stored in laboratories with five or greater specimens in storage as per the laboratory code from the cancer registry data, and with current and complete address details. In addition the whānau (extended family) of deceased participants identified as Māori from the Auckland region were requested to give consent for these specimens to be used, as per local Iwi (tribe) wishes. All participants provided written informed consent prior to specimens being requested from storage. After specimen requests were made, laboratories sent the specimens (n = 267), a copy of the pathology report, and any existing p16 slides (n = 57) to Massey University.

p16 IHC

Samples with a historic p16 slide had the existing slide used in this study. Samples were recoded and read blinded. All other samples had p16 IHC performed at a diagnostic medical laboratory (MedLab Central Ltd, Palmerston North, New Zealand). Formalin fixed paraffin embedded (FFPE) tissue was used for IHC. Sections were cut at four microns and baked onto Superfrost Plus slides at 60˚C for 60 minutes. Staining was performed on the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, USA), with antigen retrieval performed on board, using Ventana CC1 buffer for 32 minutes at 100˚C. A 1:150 dilution of the p16 (INK4a) antibody (G175-405, product code 551153) (BD Biosciences, North Ryde, Australia), was incubated on the slide for eight minutes at 36˚C. The detection of p16 was then visualised using the Ventana Optiview DAB kit (Ventana Medical Systems). A multi-tumour block positive control containing a serous ovarian carcinoma was included with each run of samples. All slides were read independently by two pathologists (BL and UV), and discordant cases reviewed to reach a consensus. Samples showing strong diffuse staining in the nucleus and cytoplasm of greater than 75% of tumour cells were considered positive. Samples showing staining in 10% to 75% of tumour cells were considered focally positive, and samples with staining in less than 10% of tumour cells were considered negative.

DNA extraction

A 10 μm slice of representative FFPE tumour block from each case with sufficient material (n = 224) was cut for DNA extraction using a new blade between each sample to avoid cross contamination of samples. DNA extractions were performed using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) as per manufacturer's instructions for tissue. A control extraction was included with each set of samples. The blank extractions were then used as negative controls in the beta globin and HPV 16 qPCRs. A pre-treatment for paraffin embedded samples was included, and a final elution volume of 100 μL was used. The quality of extracted DNA was assessed using a Nanodrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Beta-globin qPCR

The presence of amplifiable DNA was assessed on all cases with extracted DNA by qPCR targeting the human beta-globin gene using the PC03 and PC04 primers [17]. All primers used are shown in Table 1. Each 20μL reaction mix contained: 0.25 μM each primer, and 1X Fast

Table 1. Primer sequences and PCR conditions.

Primer name	Primer sequence (5'-3')	Annealing temp	Gene target/ position	Product Size (bp)	Reference
16F cloning	GAT CAG TTT CCT TTA GGT CG	62˚C	HPV 16[1]	1775	Primers designed for this project
16R cloning	GGT ACC TGC AGG ATC AGC CAT		(7014-885bp)		
PC03	ACA CAA CTG TGT TCA CTA GC	55˚C	Human beta globin gene[2]	110	Saiki et al [17]
PC04	CAA CTT CAT CCA CGT TCA CC		(827-937bp)		
GP5+	TTT GTT ACT GTG GTA GAT ACT AC	49˚C	HPV L1 gene[1]	140	de Roda Husman et al [18]
GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C		(6624-6765bp)		
16F	GTG GAC CGG TCG ATG TAT GTC T	62˚C	HPV 16 E6[1]	209	Dictor and Warrenhalt [19]
16R	TCC GGT TCT GCT TGT CCA GC		(496-704bp)		

[1] Genbank accession number: KO2718

[2] Genbank accession number: L26478

https://doi.org/10.1371/journal.pone.0186424.t001

Start SYBR Green Master (Roche Diagnostics, Basel, Switzerland). Up to 50 ng of extracted DNA was used as template. Cycling conditions were 95˚C for 10 minutes, followed by 40 cycles of: 95˚C for 15 seconds, 55˚C for 30 seconds and 72˚C for 30 seconds. PCR was followed by a melt curve from 75–85˚C with 0.2˚C increments and a three second hold. A sample was considered positive if it produced a T_m of 81.0˚C (±1.0˚C). Samples showing low amplification on the qPCR were visualised using gel electrophoresis through a 1.0% agarose gel containing 1X SYBR® Safe (Thermo Fisher Scientific) to confirm product amplification.

HPV 16 qPCR

The presence of HPV 16 was assessed on all cases with a positive beta globin qPCR result. A qPCR with melt curve analysis was developed using previously described primers [19]. Each reaction mix contained: 0.25 μM each primer, and 1X Fast Start SYBR Green Master (Roche Diagnostics). Up to 50 ng of extracted DNA was used as template. Cycling conditions were 95˚C for 10 minutes, followed by 40 cycles of: 95˚C for 15 seconds, 62˚C for 30 seconds and 72˚C for 30 seconds. PCR was followed by a melt curve from 70–80˚C with 0.2˚C increments and a five second hold. A sample was considered positive if it produced a T_m of 77.6˚C (±1.0˚C).

A positive control was made from cloned DNA of a HPV 16 positive clinical sample provided by MedLab Central Ltd (Palmerston North, New Zealand). Cloning was performed using a pGEM®-T Easy kit with JM109 High Efficiency Competent Cells (Promega, Madison, USA), according to the manufacturer's instructions with blue/white selection. The cloned plasmid contained an approximately 1.8 kb fragment of the HPV 16 genome that included the E6 and E7 gene sequence amplified using the primers detailed in Table 1. Before being used as a positive control, the cloned plasmid was subjected to automatic dye-terminator cycle sequencing with BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) to confirm genomic sequence using both the forward and reverse primers. The sequences obtained were compared using the NCBI Blast database to other published sequences available from GenBank [20].

Duplicate standard curves from eight, 10-fold serial dilutions starting from 9.56 x 10^7 copies of HPV 16 were used to determine the limit of detection of the HPV 16 qPCR.

Confirmation of non-HPV 16 types

Samples that were p16 positive / focally positive, and HPV 16 negative were subject to qPCR using the GP5+/6+ primers [18]. Each reaction contained 0.2 μM each primer, 2.0 mM $MgCl_2$, 1X PCR buffer, 0.3 mM each dNTP, 1 unit Platinum™ *Taq* (Invitrogen, Carlsbad, USA), 1.5 μM Syto® 9 (Invitrogen). Up to 50 ng extracted DNA was used as template. Cycling conditions were 95˚C for 10 minutes, followed by 40 cycles of: 95˚C for 15 seconds, 49˚C for 30 seconds and 72˚C for 30 seconds. PCR was followed by a melt curve from 70–85˚C with 0.2˚C increments and a five second hold. Known HPV 16, 18, 33, and 52 positive samples were included in each run. Samples with a T_m in the range of 76.0–81.0˚C were considered positive for HPV DNA. The GP5+/6+ PCR could not reliably discriminate HPV genotypes therefore, the product of samples positive for the GP5+/6+ PCR were purified using the PureLink® PCR purification kit (Invitrogen, Carlsbad, CA, USA). The HPV type present was then confirmed by sequencing as previously described.

Data handling and statistical analysis

The continuous variable age was categorised into a binary variable: aged 60 years or younger, and 61 years or older. Ethnicity was classified into NZ European, NZ Māori, and other.

To investigate the association between HPV positive OPC and putative risk or confounding factors (age, sex, ethnicity, and time period), each factor was tested individually for significance at $p < 0.2$ in a logistic regression model using the software package R version 3.2.0 (R Development Core team, 2010, R Foundation for Statistical Computing, Vienna, Austria). A multivariable model was built by a stepwise selection process, retaining all variables significant at $p < 0.05$ and any confounding variables. Once a main effects model was built two-way interaction terms were introduced to the model and retained if significant at $p < 0.05$.

Results

Study population

The national dataset from the cancer registry comprised a total of 621 cases from the 1996–98 (n = 113), 2003–05 (n = 185), and 2010–12 (n = 323) time periods. Diagnosis was based on histology in 94.8% (n = 589) of cases, cytology in 4.6% (n = 29) of cases and other non-specified tests in 0.6% (n = 3) of cases. The proportion of cases diagnosed on histology versus cytology was consistent over all time frames. The mean age of an OPC patient was 59.2 years. NZ Europeans made up 68.8% of the national cases, and NZ Māori a further 10.5%. There was an approximate 4:1 ratio of males to females.

Of the 344 living patients identified from the national dataset, 303 were able to be contacted, of which 52% (157/303) gave consent. Of the 157 cases, 109 specimens could be retrieved. There were 277 deceased patients identified in the national dataset. Of the 277 cases, 158 specimens were retrieved for use in the study.

Thus, the total study population comprised 267 cases with retrievable specimens, and represented all major centres. Between four and 61 cases were retrieved from each of the 15 laboratories involved. The demographic factors of the national dataset and study population were comparable as shown by the high p-values for age, sex, ethnicity and timeframe (Table 2). Therefore, the study population was considered to be reflective of the national dataset.

p16 IHC

p16 results were categorised as positive, focally positive, or negative (Fig 1). Consensus was achieved in all cases. A positive result was seen in 58.4% (156/267) of cases, 7.9% (21/267) were

Table 2. Comparison of demographic factors of the study population and national data.

		National dataset (n = 621)	Excluded cases (n = 354)	Study population (n = 267)	p-value[1]
Mean age at diagnosis		59.2 years	58.6 years	60.1 years	0.30
Sex:	Male	492 (79.2%)	283 (79.9%)	209 (78.3%)	0.82
	Female	129 (20.8%)	71 (20.1%)	58 (21.7%)	
Ethnicity:	NZ European	427 (68.8%)	237 (66.9%)	190 (71.2%)	0.77
	NZ Māori	65 (10.5%)	36 (10.2%)	29 (10.9%)	
	Other	118 (19.0%)	73 (20.6%)	45 (16.9%)	
	Not stated	11 (1.7%)	8 (2.3%)	3 (1.0%)	
Timeframe:	1996–98	113 (18.2%)	62 (17.5%)	51 (19.1%)	0.83
	2003–05	185 (29.8%)	102 (28.8%)	83 (31.1%)	
	2010–12	323 (52.0%)	190 (53.7%)	133 (49.8%)	
Deceased		277 (44.6%)	119 (33.6%)	158 (59.2%)	<0.01
Living		344 (55.4%)	235 (66.4%)	109 (40.8%)	

[1] p-value calculated compares the study population to the national dataset.

https://doi.org/10.1371/journal.pone.0186424.t002

focally positive, and 27.3% (73/267) were negative. The remaining 6.4% (17/267) of cases had no tumour present in the block (n = 10), or had insufficient material present (n = 7).

There were 57 cases with a historic p16 slide. When comparing study p16 results to original results from cases with historic p16 slides, concordance was seen in 49/49 positives, 1/2 focally positives, and 5/5 negatives. The discordant focally positive case was identified as negative by our study. The remaining case had a slide sent for the study but no p16 result in the original pathology report.

Beta-globin, HPV 16, and GP5+/6+ qPCR

DNA could be extracted from 224 samples. Following the beta-globin qPCR (Fig 2) a further 4.0% (9/224) were excluded due to a lack of amplifiable DNA. HPV16 qPCR was performed on all beta globin positive samples. Serial dilutions of a HPV 16 clone control showed the assay was capable of detecting 26 copies of the HPV 16 E6 target sequence (Fig 2). HPV 16 was detected in 74.4% (160/215) of cases.

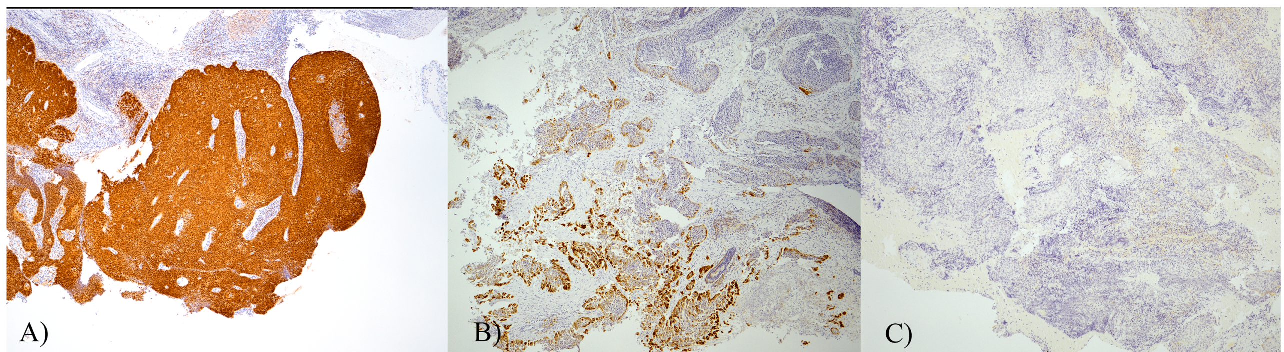

Fig 1. p16 staining patterns. A) Positive sample with >75% of tumour cells showing strong diffuse staining (magnification 40 x) B) Focally positive samples with 10–75% of tumour cells stained (magnification 40 x), and C) a negative sample with <10% of tumour cells stained (magnification 40 x).

https://doi.org/10.1371/journal.pone.0186424.g001

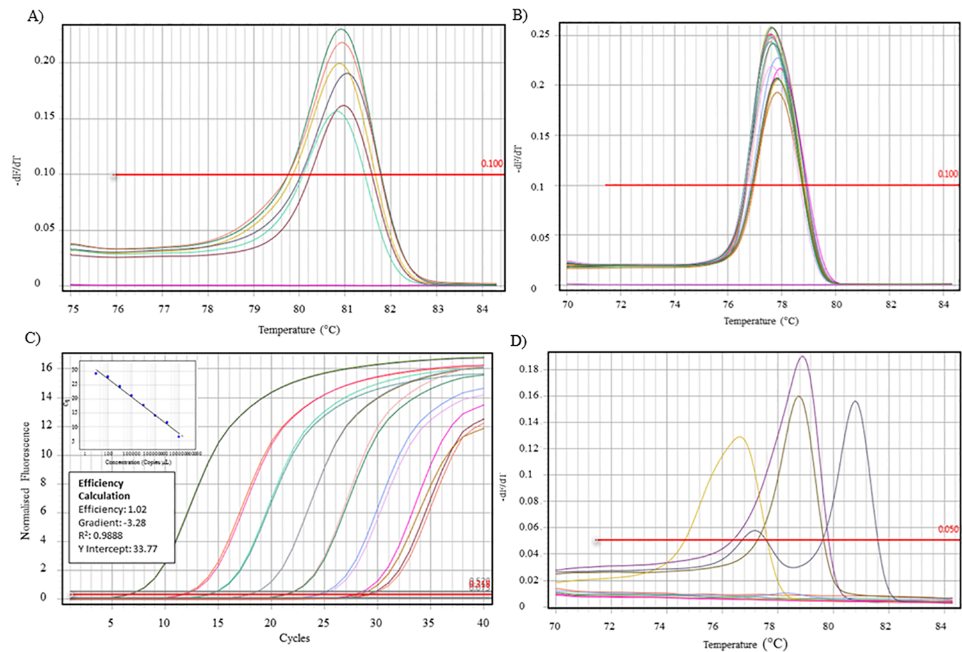

Fig 2. qPCR figure panel. A) Melt curve of beta-globin qPCR. Positive samples shown with T_m of 81.0˚C (±1.0˚C). B) Melt curve of HPV 16 qPCR. Positive samples shown with T_m of 77.6˚C (±1.0˚C). C) Standard curve of HPV 16 clone control serial dilutions. D) Melt curve of GP5+/6+ qPCR. Positive samples shown with T_m in the range of 76.0–81.0˚C.

https://doi.org/10.1371/journal.pone.0186424.g002

Results of the GP5+/6+ qPCR (n = 17) were 82% (14/17) negative, and 18% (3/17) positive (Fig 2). Of the three positive samples, HPV was confirmed by sequencing in two cases, one case each of HPV 33 and 35. The HPV type could not be detected in the remaining case. Overall, high risk HPV was detected in 75.3% (162/215) of cases. The detection of a high risk HPV type by PCR/sequencing and the p16 result is shown in Table 3.

For the purpose of further analysis, a sample is considered positive if positive by p16, and positive for either the HPV 16 qPCR, or a high risk HPV type was confirmed by sequencing. A negative sample is negative by p16, and HPV 16 qPCR negative. This approach resulted in an overall study prevalence of HPV positive OPC of 77.9% (95% CI: 71.1–83.5%). HPV 16 accounted for 98.5% of HPV positive OPC.

Statistical analysis

The proportion of cases attributable to HPV increased from 61.9% (95% CI: 40.9%– 79.2%) in 1996–98 to 63.8% (95% CI: 49.5%– 76.0%) in 2003–05, and finally to 87.5% (95% CI: 79.8%– 92.5%) in 2010–12.

Table 3. p16 result, and detection of a high risk HPV type by PCR/sequencing in archived oropharyngeal cancer biopsy samples.

		High risk HPV		Totals
		Positive	Negative	
p16 result	Positive	134	6	140
	Focally positive	11	9	20
	Negative	17	37	54
Totals		162	52	214

https://doi.org/10.1371/journal.pone.0186424.t003

Table 4. Univariable and multivariable analysis of putative risk factors associated with having an HPV positive tumour.

Variable		Univariable analysis			Multivariable analysis		
		Odds Ratio	95% CI	P value	Odds Ratio	95% CI	P value
Age	≤60	REF[1]			REF[1]		
	≥61	0.48	0.28–0.82	<0.01	0.55	0.33–0.99	0.05
Sex	Female	REF[1]			REF[1]		
	Male	1.50	0.79–2.78	0.21	1.36	0.68–2.66	0.38
Ethnicity	NZ European	REF[1]			REF[1]		
	NZ Māori	1.82	0.74–5.15	0.22	1.49	0.55–4.52	0.45
	Other	1.07	0.52–2.27	0.86	1.14	0.53–2.53	0.74
Timeframe	1996–98	REF[1]			REF[1]		
	2003–05	1.75	0.84–3.67	0.14	1.76	0.83–3.81	0.14
	2010–12	5.90	2.82–12.62	<0.01	5.65	2.60–12.30	<0.01

[1] REF is the baseline level (OR = 1.00)

https://doi.org/10.1371/journal.pone.0186424.t004

The mean age of an HPV negative patient was 66.2 years (95% CI: 65.8–66.4), compared to 56.8 years (95% CI: 56.6–56.9) for an HPV positive patient. The univariate analysis of putative risk factors showed age ($p < 0.01$) and timeframe ($p < 0.01$) to be significant (Table 4). In the final multivariable model (Table 4) there was a statistically significant association between HPV positivity and time frame (OR: 5.65, 95% CI: 2.60–12.30, 2010–12 vs 1996–98). A statistically significant association between HPV positivity and patient age was also found (OR: 0.55, 95% CI: 0.33–0.99, ≥61 years vs ≤60 years). These estimates were adjusted for sex and ethnicity.

Discussion

The increase in the proportion of HPV positive cases between 1996–98 and 2010–12 seen in this study augments Ministry of Health data showing over a three-fold increase in OPC case numbers, regardless of HPV status between 1996 and 2010. Overall, the results of this study showed that the majority of OPC cases in New Zealand are caused by HPV. An HPV positive patient is more likely to be under 60. The younger age for HPV positive patients and increase in prevalence from this study are consistent with international data [7,12,21]. Other developed counties have seen increases in the proportion of HPV positive cases ranging from 25% in Sweden between 1970 and 2007 [22], to 225% in the United States between 1988 and 2004 [23]. Moreover, there were no significant differences between the national dataset and study population in terms of patient age, sex, ethnicity, or timeframe of diagnosis, showing this study's results can be extrapolated to the wider New Zealand setting.

There was a higher proportion of deceased patients in our population than the national dataset (59% versus 45%) and this is likely due to the different consenting requirements. Due to the poorer prognosis of HPV negative tumours [13], the inclusion of a higher proportion of deceased cases may have caused an over-representation of HPV negative cases. A small regional New Zealand study performed as retrospective audit, which did not require any patient consent, showed an overall prevalence of HPV positive OPC of 63% based on p16 results alone [24]. Our overall prevalence of HPV positive cases using an accepted p16/PCR algorithm [21] was 77.9%. It is therefore unlikely that HPV negative cases were overrepresented in our study.

We requested 389 specimens from laboratories throughout New Zealand to capture our final study population of 267 cases. There were several reasons why cases were not retrieved,

such as 1) there were logistical difficulties identifying cases (due to the later introduction of computerised records in some centres, and laboratory take-overs / mergers), 2) cases were diagnosed on cytology only and had no biopsy / cell block, 3), specimens had been destroyed in the 2011 Christchurch Earthquake, and 4) specimens had been removed from storage and could not be located. Given the many reasons for samples not being included and the final comparisons of our study population to the national dataset, there was no reason to suspect any systematic bias in our sample.

In New Zealand, laboratories are required by law to report any new diagnosis of cancer to the cancer registry. This generates complete national incidence data. Our study compared only risk factors with data available from the cancer registry (age, sex, ethnicity, and timeframe of diagnosis). It is important to note these are not the only risk factors for HPV positive OPC and other significant factors such as alcohol consumption, tobacco smoking and sexual behaviours [4,11,12] could not be assessed. We therefore cannot rule out possible differences between study participants and those excluded from the study.

It should be noted, that our final criteria for a positive result was both p16 positive and either HPV 16 qPCR positive, or a high risk HPV type confirmed by sequencing. These criteria resulted in 172 results from 267 cases for analysis. This approach excluded focally positive cases and those cases that were p16 negative but HPV 16 positive by PCR. The exclusion of focally positive cases, and use of the p16 positive cut-off at 75% of tumour cells stained, aligned our definition of a p16 positive result with that used in many other studies [8–10,12,21,25].

Although p16 is routinely used alone in the diagnostic setting [26], the use of an algorithm incorporating p16 and another method (PCR or ISH) is preferred in research [8,9,21,27]. Whilst some studies only perform PCR testing on p16 positive cases [21], a more common approach is for all samples to undergo PCR testing for HPV DNA [8–10]. This approach generates four possible result categories p16 positive/HPV positive, p16 negative/HPV negative, and the equivocal results of p16 positive/ HPV negative and p16 negative/ HPV positive [28]. Discrepancies between p16 and PCR results have been reported to be between 6% and 31% [8,29]. In this study, discordant p16/PCR results were seen in 12.8% (25/195) of our cases where, eight cases were p16 positive/ high risk HPV negative, and 17 cases were p16 negative/ HPV16 positive. In the p16 positive / HPV negative cases, it is possible that p16 is upregulated by non-HPV related mechanisms [30].

The viral load in tonsillar cancer has been reported to be between 1.54×10^2 and 1.34×10^7 copies per 50 ng of DNA [31]. Our HPV 16 qPCR was capable of detecting 26 copies of HPV, therefore it is unlikely that low levels of HPV are the reason for the eight p16 positive / high risk HPV negative results. Additionally, due to using a new blade for each sample section, and the inclusion of control DNA extractions in each run it is unlikely the p16 negative / HPV positive cases are due to contamination. These 17 cases may represent a transient /bystander HPV infection unrelated to the tumour [9,28]. Junor *et al* [29] reported that p16 negative, HPV DNA positive cases were a distinct clinical entity based on survival characteristics compared to p16 positive/HPV positive and p16 negative/HPV negative cases. Evans *et al* [8] also reported intermediate survival characteristics for patients with equivocal p16/HPV results. Our criteria for a negative p16 result was less than 10% of tumour cells stained. Interestingly 88% (15/17) of the HPV DNA positive /p16 negative samples showed patchy staining in less than 10% of tumour cells.

While multi-levelled result categories or scoring systems for p16 have been reported [32–34], more frequently cases with less than 70% of tumour cells stained are regarded as negative [8,10,21]. Our study had 7.9% (21/267) focally positive p16 cases which had variable staining patterns, and the percentage of cells stained ranged from 10–80%. The single case with 80% of cells stained was defined as focally positive as staining was only present in the cytoplasm. This

is consistent with two previous studies by Chen *et al* [33] and Lewis *et al* [34] who reported 8.2% and approximately 4%, respectively, of OPC cases with partial p16 staining. The focal staining in both of these studies ranged from less than 5% of tumour cells to 90% of tumour cells, where the cases showing over 75% staining were considered focally positive based on additional criteria such as staining intensity [33,34]. Approximately half of our focally positive cases had HPV DNA detected by PCR. However, further work is required to determine if these cases contain transcriptionally active HPV and therefore are true HPV positive cases, or additional equivocal cases. Regardless, the differing published criteria and lack of international guidelines for p16 interpretation in OPC show clarity is needed. A simple cut off point may not be enough and other factors such as staining intensity, location, and confluence may need to be considered [33,34]. It is possible the age of the blocks contributed to the focally positive results in this study. When looking at each timeframe 5% from 2010–12 were focally positive, compared to 10% from 2003–05 and 15% from 1996–98. A reduced sensitivity and specificity of p16 on older specimens has been reported by Chenevert *et al* [12], however, their blocks were considerably older and dated back to 1956.

Worldwide HPV is responsible for over 90% of HPV positive OPC [7]. Co-infection is rarely described, and when present usually involves HPV 16 and another high risk type [9,10,35]. To date only HPV 16 is a recognised carcinogen in the oropharynx, while there is limited evidence for HPV 18 [6]. Co-infection was not assessed by this study. However, our findings of HPV 16 accounting for 98.5% of HPV positive cases, and the detection of HPV 33 and 35 is consistent with international data [35]. This study had one case in which the HPV type/s could not be determined. This case was p16 positive, HPV 16 negative and GP5+/6+ positive. The GP5+/6+ PCR is capable of detecting a broad spectrum of HPV types [18]. It is possible the sample contained a low risk HPV type not related to the cancer. The coincidental detection of low /intermediate risk types such as 6, 11, 32, 44, 53, 57 and 81 in OPC has been reported in a systematic review of HPV types in HNSCC by Kreimer *et al*, with HPV 6 the most commonly detected with a prevalence of 3.1% [35]. It is also possible this case contained a co-infection and therefore the types present could not be established by sequencing.

Based on our results, a HPV positive patient is more likely to be under 60, and diagnosed recently (2010–12). The prevalence of HPV positive OPC in females appears to be increasing at a faster rate than in males based on our study data. However, interpretation of this apparent trend is limited due to the low number of cases in females in this study. There were no HPV positive cases in females in 1996–98, although there was one case that was p16 positive that could not have DNA extracted. This is compared to 20% of p16/HPV DNA positive being in females in the 2010–12 time period. A statistically significant increase in New Zealand OPC cases in females of 2.1% per year between 1982 and 2010 has been previously reported [16]. Although Elwood *et al*, focused on tumour site alone without assessing HPV status, the results support the apparent increase seen in our study.

Chaturvedi *et al* [23] estimated that by 2020 the incidence of HPV positive OPC cases will outnumber the number of cervical cancer cases in the United States. Our data suggests the same scenario will likely be seen in New Zealand. The increasing prevalence of HPV positive OPC and the predominance in males supports the recent inclusion of males in the funded immunisation schedule for Gardasil ®9 in New Zealand. HPV types 16, 18, 6, 11, 31, 33, 45, 52, and 58 are included in Gardasil®9 [36]. Therefore, 99.3% of HPV positive cases with a detectable HPV type in this study contain types included in the vaccine. These results suggest that with adequate coverage, vaccination is likely to have a considerable impact on the future incidence of HPV positive OPC in New Zealand.

Supporting information

S1 File. Study database (participant demographics and lab results).
(XLSX)

Acknowledgments

We would like to thank Jackie Johnson (MedLab Central) for her work in the p16 testing and preparation of specimens for DNA extraction, and Dr Michael Dictor (Lund University Hospital, Sweden) for the HPV 33 and 52 control material. We also wish to thank the staff at laboratories throughout New Zealand who identified and retrieved cases for this study. And finally, we wish to acknowledge the patients and whānau who made this study possible by giving consent for the use of archived specimens.

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Writing – review & editing: Jackie Benschop, Bruce Lockett, Ursula van den Heever, Ruth Williams, Laryssa Howe.

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35. Kreimer AR, Clifford GM, Boyle P, Franceschi S Human papillomavirus types in head and neck squamous cell carcinomas worldwide: A systematic review.
36. Joura EA, Giuliano AR, Iversen OE, Bouchard C, Mao C, et al. (2015) A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. N Engl J Med 372: 711–723. https://doi.org/10.1056/NEJMoa1405044 PMID: 25693011

Appendix 2 - HDEC approval letter (14/STH/128)

Health and Disability Ethics Committees
C/- MEDSAFE, Level 6, Deloitte House
10 Brandon Street
PO Box 5013
Wellington

0800 4 ETHICS
hdecs@moh.govt.nz

23 October 2014

Ms Rebecca Lucas-Roxburgh
Massey University
Private Bag 11222
Palmerston North 4442

Dear Ms Lucas-Roxburgh

Re:	**Ethics ref:**	**14/STH/128**
	Study title:	Human papilloma virus (HPV) associated oropharyngeal cell carcinoma

I am pleased to advise that this application has been *approved* by the Southern Health and Disability Ethics Committee. This decision was made through the HDEC-Full Review pathway.

The Committee noted:

- Researchers don't need to put reference to funding not being achieved in the PISC.
- Researchers also do not need the final declaration by a member of the research team. The form is to be posted back so no one can attest.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the Southern Health and Disability Ethics Committee is required.

Standard conditions:

1. Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.

2. Before the study commences at *a given* locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the *Standard Operating Procedures for Health and Disability Ethics Committees* (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

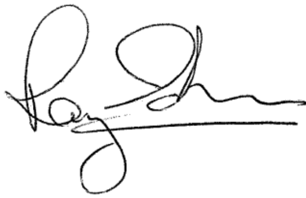
Your next progress report is due by 23 October 2015.

<u>Participant access to ACC</u>

The Southern Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC).

Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,



Ms Raewyn Idoine
Chairperson
Southern Health and Disability Ethics Committee

Encl: appendix A: documents submitted
appendix B: statement of compliance and list of members

Appendix A
Documents submitted

Document	*Version*	*Date*
Protocol	1	21 August 2014
Evidence of scientific review	1	21 August 2014
CV for CI	1	21 August 2014
CVs for other Investigators	1	21 August 2014
CVs for other Investigators	1	21 August 2014
CVs for other Investigators	1	21 August 2014
evidence of Maori consultation	1	01 September 2014
Application	1	-
Protocol	2	09 October 2014
PIS/CF	1	09 October 2014

Appendix B
Statement of compliance and list of members

Statement of compliance

The Southern Health and Disability Ethics Committee:

- is constituted in accordance with its Terms of Reference
- operates in accordance with the *Standard Operating Procedures for Health and Disability Ethics Committees*, and with the principles of international good clinical practice (GCP)
- is approved by the Health Research Council of New Zealand's Ethics Committee for the purposes of section 25(1)(c) of the Health Research Council Act 1990
- is registered (number 00008713) with the US Department of Health and Human Services' Office for Human Research Protection (OHRP).

List of members

Name	*Category*	*Appointed*	*Term Expires*
Ms Raewyn Idoine	Lay (consumer/community perspectives)	01/07/2012	01/07/2015
Mrs Angelika Frank-Alexander	Lay (consumer/community perspectives)	01/07/2012	01/07/2015
Dr Sarah Gunningham	Non-lay (intervention studies)	01/07/2012	01/07/2015
Assc Prof Mira Harrison-Woolrych	Non-lay (intervention studies)	01/09/2014	01/09/2015
Dr Fiona McCrimmon	Lay (the law)	01/09/2014	01/09/2015
Dr Nicola Swain	Non-lay (observational studies)	01/07/2012	01/07/2015
Dr Devonie Waaka	Non-lay (intervention studies)	01/07/2013	01/07/2016
Dr Mathew Zacharias	Non-lay (health/disability service provision)	01/07/2012	01/07/2015

http://www.ethics.health.govt.nz

Appendix 3 – PISCF Chapter two study

Participant Information Sheet

Your letterhead

Study title:	**Human papilloma virus (HPV) associated oropharyngeal carcinoma**		
Locality:	**Massey University, Palmerston North**	Ethics committee ref.:	**14/STH/128**
Lead investigator:	**Rebecca Lucas-Roxburgh (MSc)**	Contact phone number:	**063569099 ext 85797**

You are invited to take part in a study on HPV in oropharyngeal cancer in New Zealand. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is 5 pages long, including the Consent Form. Please make sure you have read and understood all the pages.

WHAT IS THE PURPOSE OF THE STUDY?

This study aims to determine how many cases of oropharyngeal cancer in New Zealand are caused by Human Papillomavirus (HPV), and what types of HPV are responsible. This is important as once we know how many of these cancers are caused by HPV we can then look into how to better diagnose, and even prevent them. HPV is the cause of almost all cervical cancers. We now know that HPV causes a proportion of oropharyngeal cancers (OPC). The proportion of these cancers caused by HPV varies considerably between countries and there is currently no data from New Zealand. The incidence of HPV positive OPC has increased dramatically in many developed countries over the last two decades. It is important to know which cancers are caused

by HPV as these cancers generally show improved survival for the patients, and have implications for prevention through screening and vaccination.

This project will test archived oropharyngeal biopsy specimens from three time periods (1996-98, 2003-05, and 2010-12) from all over New Zealand for the presence of HPV. The project will also find out what specific type/types of HPV are present in these samples. This testing will determine the proportion of oropharyngeal cancers caused by HPV in New Zealand and will also show any trends in the incidence of HPV positive OPC. This project will allow the burden of HPV positive OPC in New Zealand to be assessed and therefore guide future research.

Funding for this study has been provided by Massey University. The lead investigator (Rebecca Lucas-Roxburgh) is a PhD student at Massey University. Dr's Howe and Benschop are the project supervisors and are Massey University staff. Dr Lockett is the third project supervisor and he is a managing pathologist at MedLab Central. Rebecca Lucas-Roxburgh is the contact person for this study and her phone and email details are listed at the end of this information sheet.

This study will commence in October 2014 and will take approximately one year. Health and Disabilities Commission Ethics Committee (HDEC) approval has been sought and granted for this study (HDEC reference: 14/STH/128).

WHAT WILL MY PARTICIPATION IN THE STUDY INVOLVE?

You have been chosen to participate in this study due to your past diagnosis of oropharyngeal cancer (this includes cancer of the tonsil, base of tongue, and oropharynx). The information about your diagnosis was retrieved from the New Zealand cancer registry after this study gained ethical approval.

This study will use a stored biopsy sample, taken at the time of your diagnosis. On this sample we will perform a test that suggests the presence of HPV. If this first test is positive other tests will be done to confirm the presence and type of HPV. These tests will only be done if you choose to participate in the study. If you do not wish to participate your samples will remain stored at the laboratory where your diagnosis happened.

Participation in this study involves giving your consent for us to use your stored sample by posting the attached consent form using the envelope provided. There are no questionnaires or interviews that need to be completed.

If you chose to participate some of your health information will be used by the research team. This information is collected by the New Zealand Cancer Registry and includes information about your details (such as NHI number and ethnicity) and your tumour (such as its grade and stage). This information will be stored in an anonymous form and no information that could identify you will ever be used when the results of this study are published.

WHAT ARE THE POSSIBLE BENEFITS AND RISKS OF THIS STUDY?

There are no significant risks associated with participation in this study. We are using samples that have already been taken, therefore there are no procedures that could cause discomfort or lead to any side-effects for you.

The benefit of participating in this study is to increase our health knowledge. This study will provide data on the proportion of oropharyngeal cancers in New Zealand caused by HPV. This new knowledge is important as once we know the burden of these cancers we can then look forward into how to better diagnose, and even prevent them.

WHO PAYS FOR THE STUDY?

This study will be funded by various grants. You will not incur any costs by participating in this study. The addressed postage paid envelope is included to return your consent form if you wish to participate.

WHAT ARE MY RIGHTS?

Participating in this study is your choice. Feel free to discuss your potential participation with whanau / friends before completing the consent form. You are free to decline to participate in this study, or withdraw from the research. If you give consent then at any time wish to withdraw from participating please make contact with the lead investigator using the details at the end of this form.

As a participant you have the right to access any information that we collect about you. The type of information collected is described above in ‘what will my participation in the study involve’.

Any information about you will only be able to be accessed by the research team. All information will be coded to make it anonymous. Hard copy information will be stored in a locked filing cabinet and any digital information will be stored in a password protected computer file. No information that could identify you will ever be released as part of the findings of this research.

WHAT HAPPENS AFTER THE STUDY OR IF I CHANGE MY MIND?

Any data generated by this study will be stored in an anonymous form for at least three years so it is available to guide future research. The lead investigator is responsible for the secure storage of data and as mentioned above this involves the use of lockable filing cabinets and password protected computer files.

After completion of the study the samples used in this study will be returned to the laboratory they were retrieved from.

Once the study is complete results will be communicated at scientific conferences, and by publication in scientific journals. Participants are able to request a summary of the study’s findings by ticking the box on in the attached consent form. It is estimated that this study will take around one year. The summary of results is therefore likely to be made available by December 2015.

WHO DO I CONTACT FOR MORE INFORMATION OR IF I HAVE CONCERNS?

If you have any questions, concerns or complaints about the study at any stage, you can contact:

Rebecca Lucas-Roxburgh, PhD student

Phone: 06 356 9099 extension 85797

Email: R.Lucas-Roxburgh@massey.ac.nz

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

Phone: 0800 555 050
Fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

For Maori health support please contact :

Dr Maureen Holdaway, Deputy Director of Maori Health and Development, College of Health, Massey University

Phone: 06 356 9099 extension 85092

Email: M.A.Holdaway@massey.ac.nz

You can also contact the health and disability ethics committee (HDEC) that approved this study on:

Phone: 0800 4 ETHICS

Email: hdecs@moh.govt.nz

Your letterhead

Consent Form

If you need an INTERPRETER, please tell us.

Please tick to indicate you consent to the following

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet.	Yes ☐	No ☐
I have been given sufficient time to consider whether or not to participate in this study.	Yes ☐	No ☐
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	Yes ☐	No ☐
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	Yes ☐	No ☐
I consent to the research staff collecting and processing my information, including information about my health.	Yes ☐	No ☐
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes ☐	No ☐
I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	Yes ☐	No ☐
I know who to contact if I have any questions about the study in general.	Yes ☐	No ☐
I understand my responsibilities as a study participant.	Yes ☐	No ☐
I wish to receive a summary of the results from the study.	Yes ☐	No ☐

Declaration by participant:

I hereby consent to take part in this study.

Participant's name:

Signature: Date:

Appendix 4 – Beta-Globin qPCR SOP

Beta-globin PCR

Nucleic acid extraction	Kit
DNA	Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required)
RNA(+RT-PCR)	Qiagen RNeasy MiniKit or RNeasy FFPE Kit

Primers	Name	Sequence (5'-3')	Size	Position[1]	Target
Forward	PC03	ACA CAA CTG TGT TCA CTA GC	110	827-847	Human Beta-globin gene
Reverse	PC04	CAA CTT CAT CCA CGT TCA CC		917-937	

[1] Based on Human Beta-Globin gene sequence (Genbank accession number: L26478)

PCR kit: Roche FastStart SYBR Green mastermix

Reagent mix	Volume (20 µL)
Sterile distilled water	Variable, DNA volume dependent
2x SYBR green mastermix	10.0 µL
10 µM 16F primer	0.5 µL
10 µM 16R primer	0.5 µL
DNA	Up to 50ng

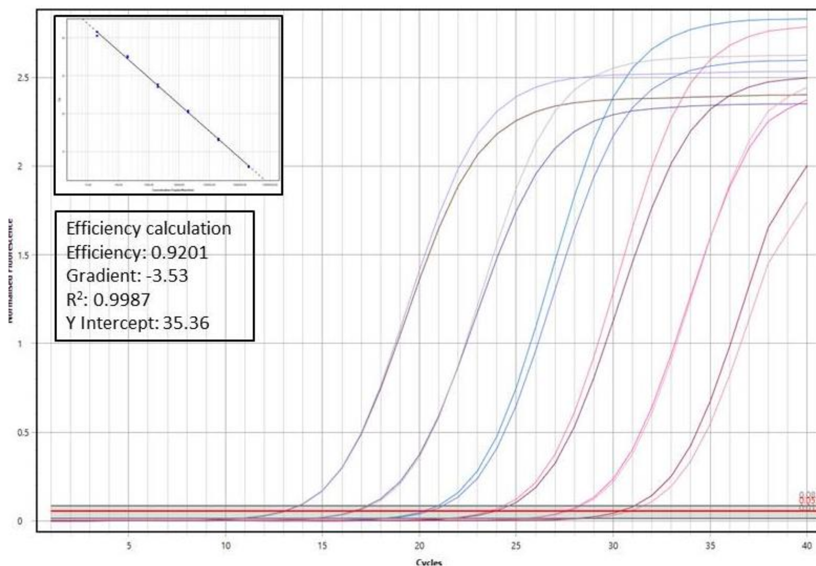
PCR controls	Description
Positive	Beta-globin clone control (Plasmid with 665 bp fragment of the human Beta-globin gene)
Negative	Control/Blank DNA extraction
Non-template control	Nuclease free water

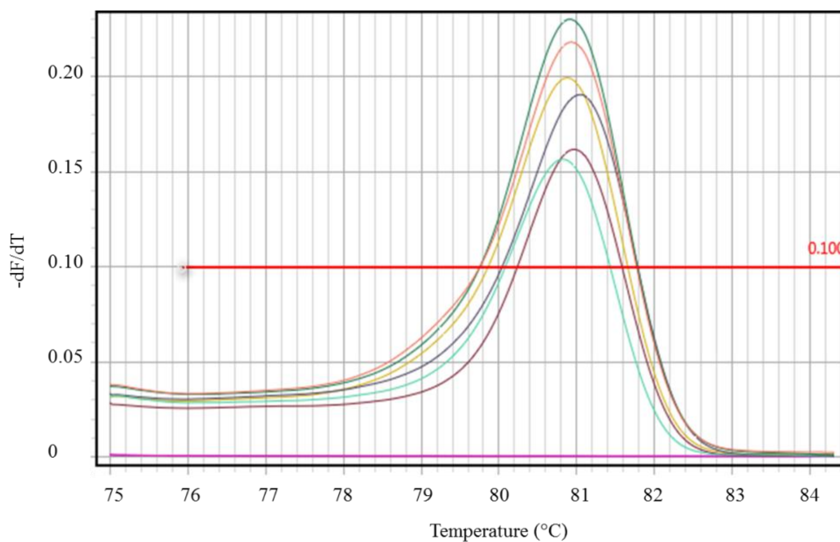
PCR program name: Beta-globin

Cycling parameters:	Temp (°C)	Time	No. cycles
Hold	95	10 min	1
Denature	95	15 sec	40
Anneal	55	30 sec	
Extension	72	30 sec	
Melt	75-85 (0.2 increments)	Three second hold	1

Electrophoresis	**Description**	**Size of amplicon(s) (bp)**
Agarose gel MW marker	1.5% 100 bp	110

Standard curve and melt images
Assay capable of detecting 13 copies of the target sequence. T_m : 81.0°C (±1.0°C).





References
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., . . . Erlich, H. A. (1988). Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase, 487-491.

Appendix 5 - HPV 16 qPCR SOP

HPV 16 PCR

Nucleic acid extraction	Kit
DNA	Qiagen DNeasy Blood and Tissue kit with FFPE pre-treatment step

Primers	Name	Sequence (5'-3')	Size	Position[1]	Target
Forward	16F	GTG GAC CGG TCG ATG TAT GTC T	209	496-519	HPV 16 E6/E7 gene
Reverse	16R	TCC GGT TCT GCT TGT CCA GC		685-704	

[1] Based on HPV 16 sequence (Genbank accession number: KO2718)

PCR kit: Roche FastStart SYBR Green mastermix

Reagent mix	Volume (20 µL)
Sterile distilled water	Variable, DNA volume dependent
2x SYBR green mastermix	10.0 µL
10 µM 16F primer	0.5 µL
10 µM 16R primer	0.5 µL
DNA	Up to 50ng

PCR controls	Description
Positive	16-1 clone control (Plasmid with ~1.8 kb fragment of the HPV 16 genome)
Negative	Control/Blank DNA extraction
Non-template control	Nuclease free water

PCR program name: HPV 16

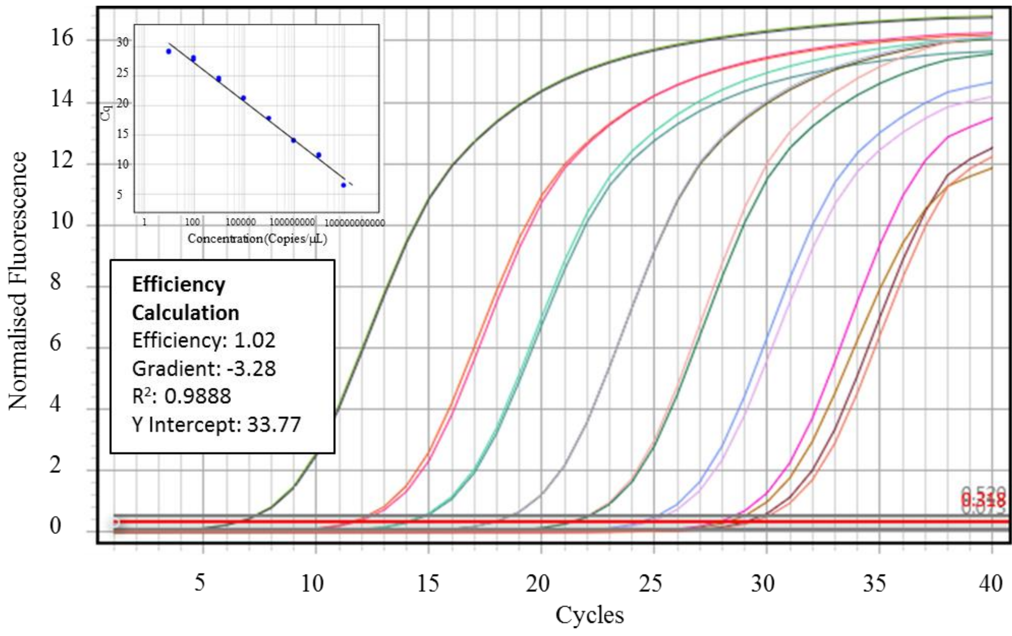
Cycling parameters:	Temp (°C)	Time	No. cycles
Hold	95	10 min	1
Denature	95	15 sec	40
Anneal	62	30 sec	
Extension	72	30 sec	
Melt	70-80 (0.2 increments)	Five second hold	1

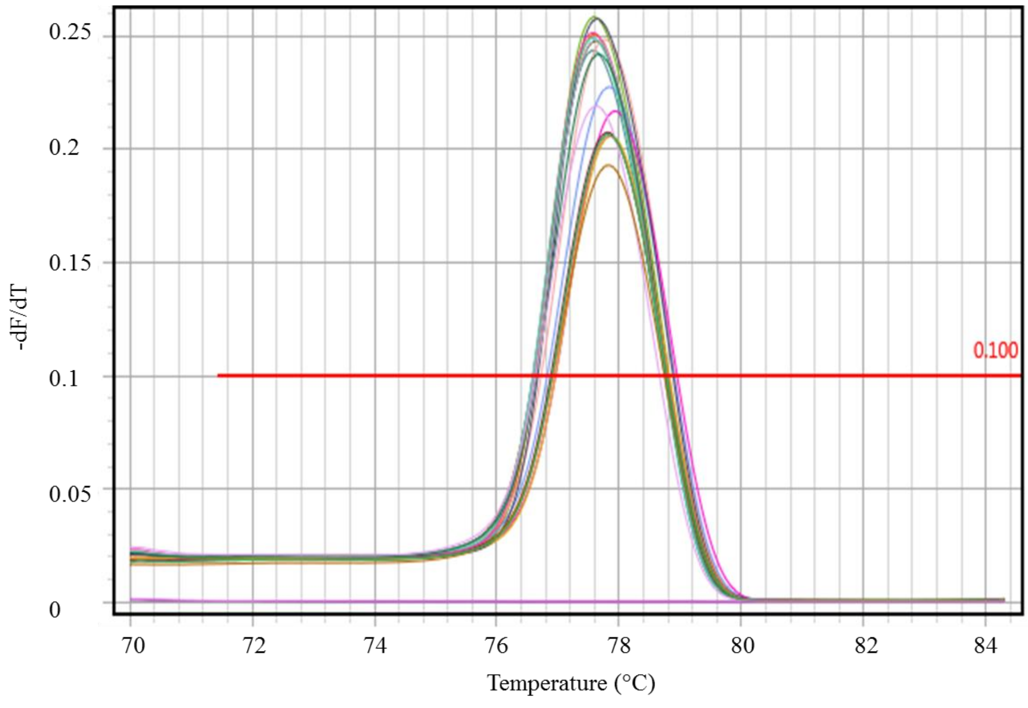
Electrophoresis	Description	Size of amplicon(s) (bp)
Agarose gel	1.5%	209
MW marker	100 bp	

Standard curve and melt images

Assay capable of detecting 26 copies of the target sequence.

T_m : 77.6°C (±1.0°C).





References

Dictor, M., & Warenholt, J. (2011). Single-tube multiplex PCR using type-specific E6/E7 primers and capillary electrophoresis genotypes 21 human papillomaviruses in neoplasia. *Infect Agent Cancer, 6*(1), 1. doi: 10.1186/1750-9378-6-1

Appendix 6 – GP5+/6+ qPCR SOP

HPV GP5+/6+ PCR

Nucleic acid extraction	Kit
DNA	Qiagen DNeasy Blood and Tissue kit with FFPE pre-treatment step

Primers	Name	Sequence (5'-3')	Size	Position[1]	Target
Forward	GP5+	TTT GTT ACT GTG GTA GAT ACT AC	140	6624-6646	HPV L1 gene
Reverse	GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C		6741-6765	

[1] Based on HPV 16 sequence (Genbank accession number: KO2718)

PCR kit: Invitrogen Platinum Taq Polymerase

Reagent mix	Volume (20 µL)
Sterile distilled water	Variable, DNA volume dependent
10x PCR buffer	2.0 µL
$MgCl_2$	0.8 µL
dNTPs	0.6 µL
10 µM GP5+ primer	0.4 µL
10 µM GP6+ primer	0.4 µL
Taq	0.2 µL
Syto® 9	0.6 µL
DNA	Up to 50ng

PCR controls	Description
Positive	Known positive clinical samples for HPV types 16, 18, 33 and 52. And 18/52 co-infected sample (367376)
Negative	Control/Blank DNA extraction
Non-template control	Nuclease free water

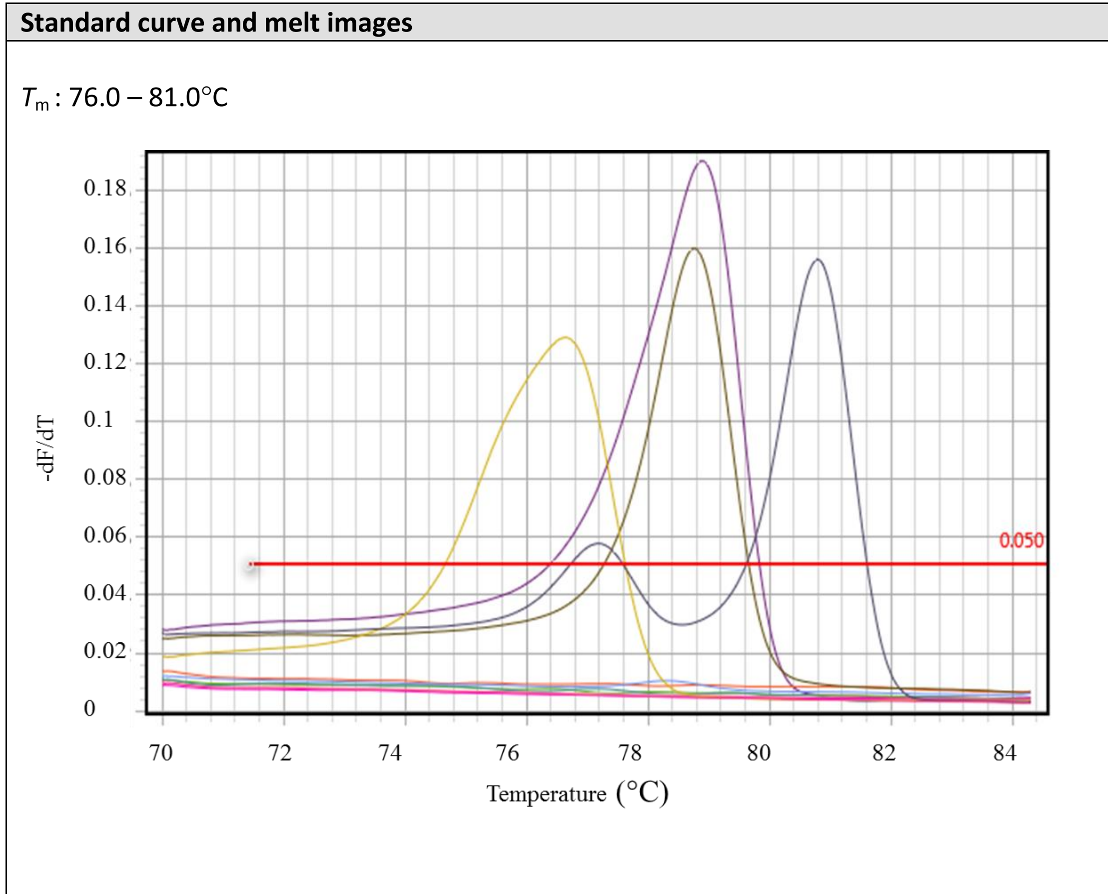
PCR program name: GP5/6

Cycling parameters:	Temp (°C)	Time	No. cycles
Hold	95	10 min	1
Denature	95	15 sec	40
Anneal	49	30 sec	
Extension	72	30 sec	
Melt	70-85 (0.2 increments)	Five second hold	1

Electrophoresis	Description	Size of amplicon(s) (bp)
Agarose gel MW marker	1.5% 100 bp	140

Standard curve and melt images

T_m : 76.0 – 81.0°C



References

de Roda Husman, A. M., Walboomers, J. M., van den Brule, A. J., Meijer, C. J., & Snijders, P. J. (1995). The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *The Journal Of General Virology, 76 (Pt 4)*, 1057-1062.

Appendix 7 – HPV 16 E7 qPCR SOP

HPV 16 E7 PCR

Nucleic acid extraction	Kit
DNA	Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required)
RNA(+RT-PCR)	Qiagen RNeasy MiniKit or RNeasy FFPE Kit

Primers	Name	Sequence (5'-3')	Size	Position[1]	Target
Forward	E7F	ACA CAA CTG TGT TCA CTA GC	75	827-847	Human Beta-globin gene
Reverse	E7R	CAA CTT CAT CCA CGT TCA CC		917-937	

[1] Based on Human Beta-Globin gene sequence (Genbank accession number: L26478)

PCR kit: Roche FastStart SYBR Green mastermix

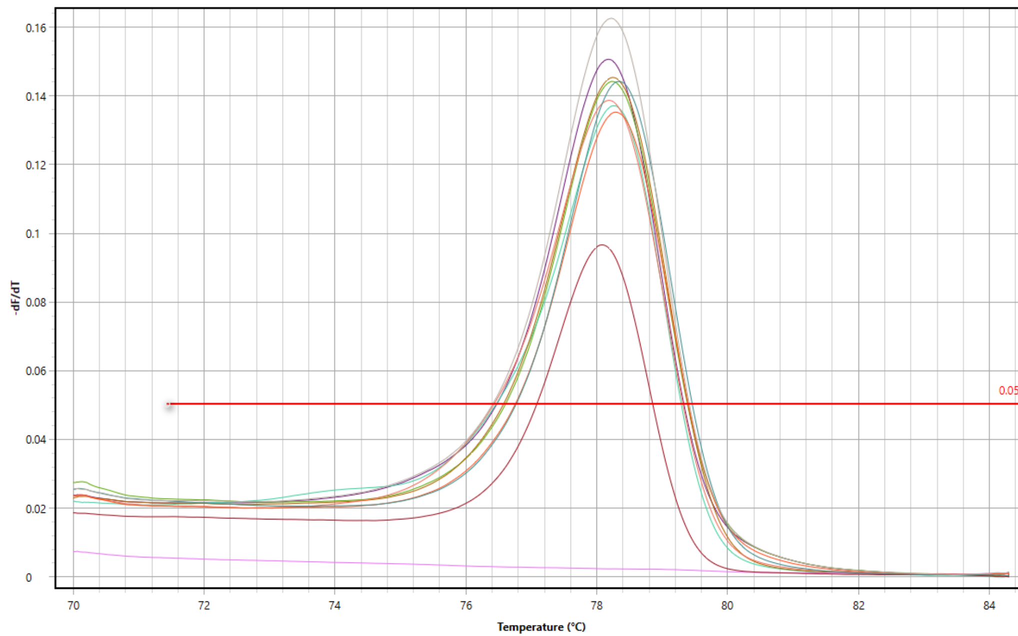
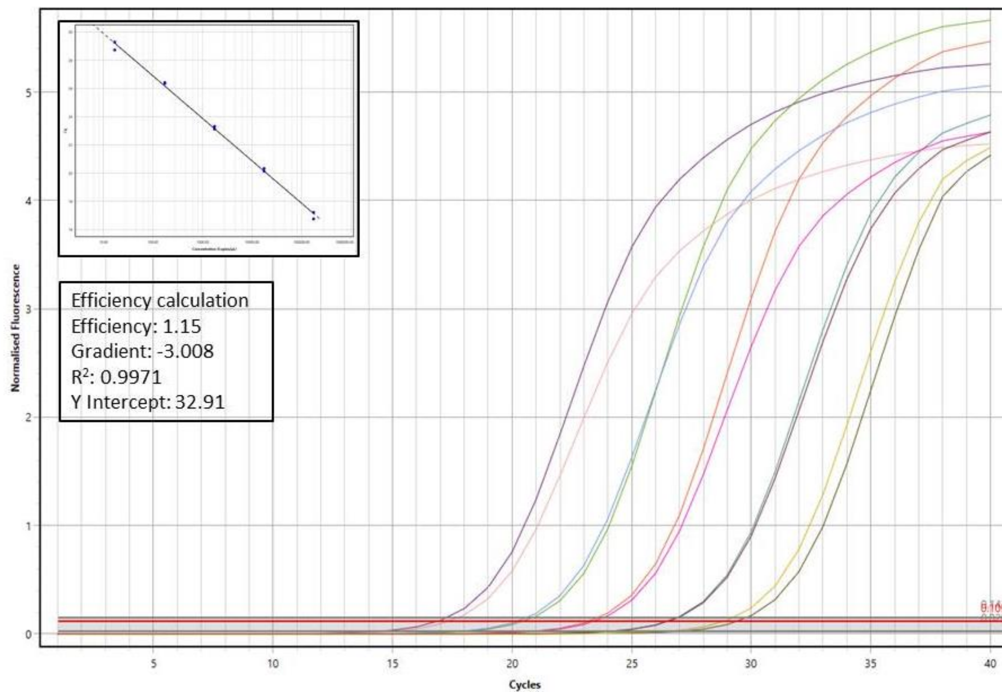
Reagent mix	Volume (20 µL)
Sterile distilled water	Variable, DNA volume dependent
2x SYBR green mastermix	10.0 µL
10 µM 16F primer	0.3 µL
10 µM 16R primer	0.3 µL
DNA	Up to 50ng

PCR controls	Description
Positive	Beta-globin clone control (Plasmid with 665 bp fragment of the human Beta-globin gene)
Negative	Control/Blank DNA extraction
Non-template control	Nuclease free water

PCR program name: Beta-globin

Cycling parameters:	Temp (°C)	Time	No. cycles
Hold	95	10 min	1
Denature	95	15 sec	40
Anneal	55	30 sec	
Extension	72	30 sec	
Melt	75-85 (0.2 increments)	Three second hold	1

Electrophoresis	Description	Size of amplicon(s) (bp)
Agarose gel	1.5%	110
MW marker	100 bp	

Standard curve and melt images
Assay capable of detecting 13 copies of the target sequence. T_m : 78.5°C (±1.0°C).  

References
Lamarcq, L., Deeds, J., Ginzinger, D., Perry, J., Padmanabha, S., & Smith-McCune, K. (2002). Regular Articles: Measurements of Human Papillomavirus Transcripts by Real Time Quantitative Reverse Transcription-Polymerase Chain Reaction in Samples Collected for Cervical Cancer Screening. *The Journal of Molecular Diagnostics, 4*, 97-102. doi: 10.1016/S1525-1578(10)60687-3

Health and Disability Ethics Committees
Ministry of Health
Freyberg Building
20 Aitken Street
PO Box 5013
Wellington
6011

0800 4 ETHICS
hdecs@moh.govt.nz

02 November 2015

Ms Rebecca Lucas-Roxburgh
Massey University
Private Bag 11222
Palmerston North 4442

Dear Ms Lucas-Roxburgh

Re:	**Ethics ref:**	**15/NTB/155**
	Study title:	Early detection markers and risk factor analysis for Human papillomavirus associated oropharyngeal cancer

I am pleased to advise that this application has been *approved* by the Northern B Health and Disability Ethics Committee. This decision was made through the HDEC-Full Review pathway.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the Northern B Health and Disability Ethics Committee is required.

Standard conditions:

1. Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.

2. Before the study commences at *a given* locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the *Standard Operating Procedures for Health and Disability Ethics Committees* (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

Your next progress report is due by 01 November 2016..

Participant access to ACC

The Northern B Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC).

Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,

Raewyn Sporle
Chairperson
Northern B Health and Disability Ethics Committee

Encl: appendix A: documents submitted
appendix B: statement of compliance and list of members

Appendix A
Documents submitted

Document	*Version*	*Date*
Protocol: Study Protocol	1	19 August 2015
Survey/questionnaire: working draft of questionnaire	1	19 August 2015
CV for CI: Lucas-Roxburgh CV	1	19 August 2015
CVs for other Investigators: Dr Benschop CV	1	19 August 2015
CVs for other Investigators: Dr Lockett CV	1	19 August 2015
Evidence of scientific review	1	19 August 2015
Evidence of Maori consultation - Dr Holdaway, Massey University	1	19 August 2015
Evidence of Maori Consultation - Dr Wihongi, Waitemata DHB	1	19 August 2015
Evidence of Maori consultation - Rika-Heke, CMDHB	1	19 August 2015
PIS/CF: PISCF	1	19 August 2015
CVs for other Investigators: Dr Howe CV	1	19 August 2015
Covering Letter: letter of reply, re: provisional response	1	02 October 2015
PIS/CF: updated PISCF	2	02 October 2015
Protocol: updated study protocol	2	02 October 2015
covering letter to be sent with questionnaire	1	02 October 2015

Appendix B
Statement of compliance and list of members

Statement of compliance

The Northern B Health and Disability Ethics Committee:

- is constituted in accordance with its Terms of Reference
- operates in accordance with the *Standard Operating Procedures for Health and Disability Ethics Committees*, and with the principles of international good clinical practice (GCP)
- is approved by the Health Research Council of New Zealand's Ethics Committee for the purposes of section 25(1)(c) of the Health Research Council Act 1990
- is registered (number 00008715) with the US Department of Health and Human Services' Office for Human Research Protection (OHRP).

List of members

Name	*Category*	*Appointed*	*Term Expires*
Mrs Raewyn Sporle	Lay (the law)	01/07/2012	01/07/2015
Mrs Maliaga Erick	Lay (consumer/community perspectives)	01/07/2012	01/07/2015
Mrs Phyllis Huitema	Lay (consumer/community perspectives)	19/05/2014	19/05/2017
Dr Nora Lynch	Non-lay (health/disability service provision)	01/07/2015	01/07/2018
Miss Tangihaere Macfarlane	Lay (consumer/community perspectives)	19/05/2014	19/05/2017
Mrs Kate O'Connor	Non-lay (other)	01/07/2012	01/07/2015
Mrs Stephanie Pollard	Non-lay (intervention studies)	01/07/2012	01/07/2015

Unless members resign, vacate or are removed from their office, every member of HDEC shall continue in office until their successor comes into office (HDEC Terms of Reference)

http://www.ethics.health.govt.nz

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

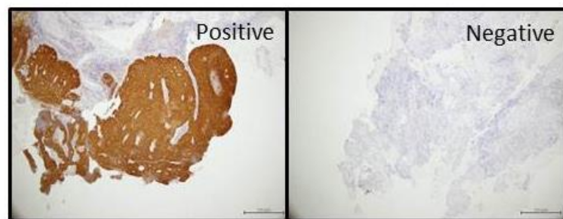
Human papilloma virus (HPV) associated oropharyngeal carcinoma: Summary of results

Background:
HPV is the cause of almost all cervical cancers. We now know that HPV causes a proportion of oropharyngeal cancers (OPC). The proportion of these cancers caused by HPV varies between countries and there is currently no data from New Zealand.

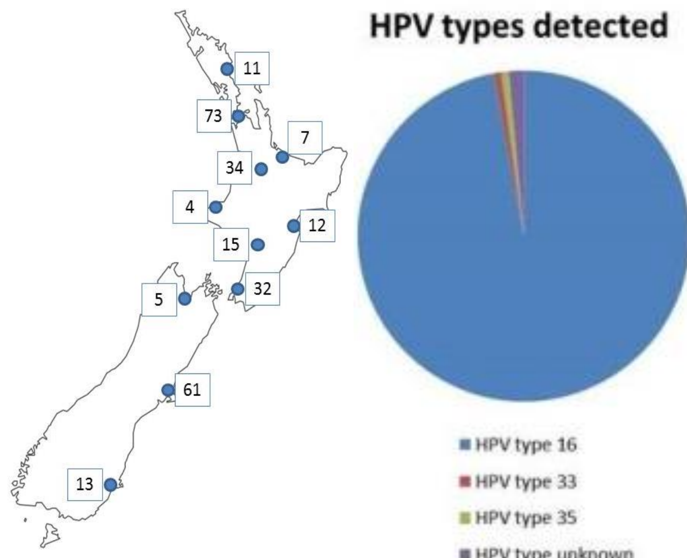
Project Aim:
This study sought to determine how many cases of oropharyngeal cancer in New Zealand were caused by HPV over the periods 1996-98, 2003-05 and 2010-12, and what types of HPV are responsible.

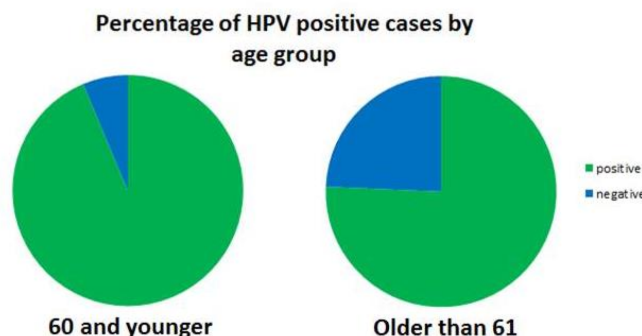
Methods:
- Health and Disabilities Commission Ethics Committee (HDEC) approval was granted for this study (Reference: 14/STH/128).
- All samples had a p16 test. This test detects a protein which is often seen when the HPV virus is the cause of the tumour.
- We then used DNA based tests to see if samples were HPV positive, and what types of HPV were present.

Results:
- 267 cases from around New Zealand were included in this study (see map). These cases were reflective of the national cases.
- Overall, 77.9% of cases were HPV positive.
- The percentage of HPV positive cases increased from 61.9% in 1996-98 to 87.5% in 2010-12.
- Of the HPV positive cases, 97% were HPV type 16, 1% was HPV type 33, and 1% HPV type 35. The HPV type could not be determined in 1% of cases.



Microscope view of two biopsies after p16 test. A positive result is shown by the presence of a dark brown colour (left), compared to no brown colour in a negative result (right).





Results (continued):
- The average age of an HPV positive patient was 56.8 years, compared to 66.2 years for an HPV negative patient.
- There was no association between HPV positive OPC and sex, or ethnicity of the patient.

What does this mean for me?
Allowing us to use your sample for this research has helped improve our knowledge of HPV in OPC in New Zealand.

What does this mean for OPC in New Zealand?
This study has shown the majority of cases of OPC are caused by HPV, and the number of cases is increasing.

New Zealand has recently introduced a new cervical cancer vaccine (Gardasil® 9) for those aged 9 to 26. This vaccine protects against nine types of HPV. It is possible in the future that almost all HPV positive OPC cases could be prevented by this vaccine.

We are currently in the process of submitting our results for publication in a scientific journal. If you would like a copy of the full article please email us: R.Lucas-Roxburgh@massey.ac.nz

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Thank you again for your participation in this study.

Appendix 9 – Participant summary of results

Appendix 10 – Invitation to participate

[Date]

Dear [Insert name],

Thank you for agreeing to be part of our study "Human papillomavirus (HPV) associated oropharyngeal squamous cell carcinoma" (HDEC reference: 14/STH/128) looking at HPV in oropharyngeal cancer (OPC) in New Zealand. The process of retrieving the specimens took longer than anticipated, but we have now completed the study and our results provide a representative picture of how many cases of oropharyngeal cancer in New Zealand are caused by HPV. Enclosed is a summary of those results, as requested when you signed up for the study.

Now we know HPV causes a significant proportion of OPC we are doing more research looking at the risk factors for HPV positive versus negative OPC. I have included an information sheet and questionnaire for this new study titled "Risk factor analysis for human papillomavirus associated oropharyngeal cancer" (HDEC reference: 15/NTB/155) and invite you to take part. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect any medical care you may receive. If you do want to take part now, but change your mind later, you can pull out of the study before its results are published. Before you decide if you want to participate you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this. If we don't receive a questionnaire back from you, we will assume you do not wish to participate, and we will not contact you again.

If you want to participate, the questionnaire will take around 20 minutes to complete. You can complete this online by going to: **https://massey.au1.qualtrics.com/jfe/form/SV_6r4OSw0DOAYT63H**

and using **[insert ref #]** as your reference number, or by filling in the hard copy and returning it using the envelope provided. All of your responses to this questionnaire are confidential and nothing that could identify you will ever be used when the results of this study are published. When you return the questionnaire the reference number on it will be matched to your study number from the original study. We will not match this number to any personal details such as name, address, or NHI.

I understand your diagnosis was some time ago and remembering some of the things we ask may be difficult. Please just complete the questionnaire as best you can. The questionnaire covers some potentially sensitive topics. We appreciate you answering the questions as honestly as possible; this will help us improve our knowledge of HPV positive oropharyngeal cancers.

If have any questions about the study results or participating in the new study, please contact me using the details below.

Your participation is greatly appreciated.

Yours sincerely,

Rebecca Lucas Roxburgh BMLS MSc

PhD Student, Massey University

Phone: 06 356 9099 xt 85797

Email: R.Lucas-Roxburgh@massey.ac.nz

Appendix 11 Questionnaire development

Questionnaire development

The questionnaire was developed with input from the research team, and Ear, Nose and Throat (ENT) surgeons Dr Julian White and Dr Aneesh Kumar. The questionnaire was designed to include a combination of previously reported risk factors and a broader more exploratory section for other possible factors such as: factors that are linked with other cancers (eg. NSAID use), other conditions that have increased in the same timeframe as HPV positive OPC (eg. allergies), other comorbidities / significant medical conditions and questions on what symptoms (if any) were experienced with a goal to direct possible earlier detection tests.

As much as possible questions were taken from other existing questionnaires or the literature. The sources for questions are outlined below.

Section A:

Question 4: Ethnicity taken from statistics New Zealand 2013 census

Question 5: Income based on New Zealand tax codes from IRD website, with the addition of pensions and benefits.

Section B:

Questions 11-13 (and 15-17 for previous drinking) from: http://www.niaaa.nih.gov/research/guidelines-and-resources/recommended-alcohol-questions

Questions 18 and 19 were from the 2013 New Zealand census, which also defined regular smoking as more than one per day.

Questions 23-25_from Kerssemakers (2000) in European monitoring centre for drugs and drug addiction: A cannabis reader: global issues and local experiences. The category of used less than five times in my life was included to ensure those with only minor experimental use were not counted as regular users.

Section C:

Intervals for questions 27/28 and 30/31 originally taken from: Rautava J, Willberg J, Louvanto K, Wideman L, Syrjänen K, Grénman S*, et al.* Prevalence, genotype distribution and persistence of human papillomavirus in oral mucosa of women: a six-year follow-up study. PloS one 2012;7(8):e42171-e doi 10.1371/journal.pone.0042171.

Section D:

List of symptoms for question 50 taken from: Clark D. Beyond cervical cancer: Human papillomavirus (HPV) and its role in oropharyngeal squamous cell carcinoma. Canadian Journal of Dental Hygiene 2013;47(3):135-8.

Pilot testing

After development, the questionnaire was tested using a group of six previous OPC patients. These patients had made contact with us and offered further assistance after being invited to participate in the Chapter two study. The group comprised of four males and two females, with an age range from early-40s to mid-70s. Pilot testing was by phone interview and those piloting the questionnaire were asked to look at the questions with the following in mind: Am I sure what the question is asking? Could I come up with an answer to this question? Or how do I feel about this question? In addition to any general comments/suggesting or things they felt should be included.

After pilot testing the following changes were made to the questionnaire:

1. The ranges for sexual partners were expanded and the upper range set at >50 partners
2. Further explanations of what constituted smoking were added
3. Questions on heavier drinking at any earlier age were included
4. Rheumatic fever was added to the list of conditions in question 28
5. Questions on lifetime heavier use of aspirin or NSAIDs were added
6. An unusual smelling body odour/sweat was added to question 50.

The question on income was retained although it was the question with most objections from pilot testing. Justification for its inclusion was the comparison to international work which describes HPV positive cancers in those of higher income/ higher socioeconomic status (D'Souza G, Zhang HH, D'Souza WD, Meyer RR, Gillison ML. Moderate predictive value of demographic and behavioral characteristics for a diagnosis of HPV16-positive and HPV16-negative head and neck cancer. Oral oncology 2010;46(2):100-4 and Krupar R, Hartl M, Wirsching K, Dietmaier W, Strutz J, Hofstaedter F. Comparison of HPV prevalence in HNSCC patients with regard to regional and socioeconomic factors. European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies 2014;271(6):1737-45)

Further modifications

Occupation and exposures questions

After recruitment of 18 participants, it was observed that three had mentioned they were welders. This was from discussions with two local patients at enrolment, and one

comment in a returned questionnaire from Auckland. Following this observation, questions seven to ten on occupation and exposure to hazardous substances were added.

Retrospective recruitment

In order to achieve the desired sample size it was necessary to include a group of participants recruited retrospectively who had previously been involved in Chapter two research. As the time since diagnosis was up to 19 years for this group some questions needed to be reworded to account for this. The modified questionnaire used for this participant group is included in appendix 13.

Appendix 12 – Participant questionnaire

Human Papillomavirus (HPV) associated oropharyngeal carcinoma

Participant Questionnaire

All responses to this questionnaire are STRICTLY CONFIDENTIAL and available only to the researchers.

Nothing that could identify you will ever be used when the results of this study are published.

- **The purpose of this questionnaire is to find out more about the risk factors for HPV positive oropharyngeal cancer. Many questions are based on risk factors reported by overseas studies.**
- **You may think some of the questions ask for sensitive information. It is your choice whether you choose to answer any question in the questionnaire.**
- **All the questions included will help improve our understanding of oropharyngeal cancer and we appreciate you taking the time to answer the questions as honestly as possible.**
- **There is a comments section at the end of the questionnaire, feel free to add in any extra information you feel may be useful for the study.**
- **If you have any queries about any part of this questionnaire please contact Rebecca Lucas-Roxburgh**
    - **Phone: 06 356 9099 extension 85797**
    - **Email: R.Lucas-Roxburgh@massey.ac.nz**

Section A: General information

1. Are you?

☐ Male
☐ Female

2. What age group do you fit into?

☐ 40 years or younger
☐ 41 – 45 years old
☐ 46 – 50 years old
☐ 51 – 55 years old
☐ 56 – 60 years old
☐ 61 – 65 years old
☐ 66 – 70 years old
☐ 71 years or older

3. Which one of these statements best describes your marital status?

☐ Single
☐ I am married / in a civil union / with a de facto partner
☐ In a relationship

4. **Which ethnic groups do you belong to?** (tick all that apply)
   - ☐ New Zealand European
   - ☐ Other European
   - ☐ Maori
   - ☐ Samoan
   - ☐ Cook Island Maori
   - ☐ Tongan
   - ☐ Niuean
   - ☐ Chinese
   - ☐ Indian
   - ☐ Other (please state) ________________________

5. **Which statement best describes your total yearly income (before tax) for the last 12 months?** (tick all that apply)
   - ☐ Zero income
   - ☐ Up to $14,000
   - ☐ From $14,001 to $48,000
   - ☐ From $48,001 to $70,000
   - ☐ $70,001 and over
   - ☐ Received Pension (New Zealand superannuation, or equivalent overseas pension)
   - ☐ Received work and income benefit (either job seeker support, sole parent support or supported living payment)

6. **What is your highest qualification?**
   - ☐ None
   - ☐ High school qualification (such as NCEA levels 1-3, school certificate, sixth form certificate, higher leaving certificate, university entrance)
   - ☐ University qualification (degree, diploma, certificate)
   - ☐ Postgraduate degree
   - ☐ Trade Certificate
   - ☐ Other (please state) ___________________________

7. **What is your occupation?____**

8. **If you have had other occupations please list them here:**

____

____

9. **During your working life, have you been exposed to potentially harmful substances (eg. chemicals, fumes, gas, dust, solvents, acids, pesticides, asbestos)**
   - ☐ Yes
   - ☐ No (go to question 11)

10. **Please tell us more about what you were exposed to:**

   - Type of substance:____
   - How long you were exposed for:____
   - Was any safety equipment used (eg. Masks, gloves etc): ____________________

<u>Section B: Alcohol use and smoking history</u>

11. During the last 12 months, on average, how often did you have an alcoholic drink?

☐ Daily
☐ 3 to 6 times a week
☐ 1 to 2 times a week
☐ 2 to 3 times a month
☐ Less than once a month
☐ I do not drink alcohol (go to question 14)

12. During the last 12 months, how many alcoholic drinks did you have on a typical day when you drank alcohol?

Note: a drink is defined as one can of beer (330 mL), one glass of wine (100 mL), or 30 mL of straight spirits.

☐ 12 or more drinks
☐ 9 to 11 drinks
☐ 7 to 8 drinks
☐ 5 to 6 drinks
☐ 3 to 4 drinks
☐ 1 to 2 drinks

13. During the last 12 months, what is the largest number of alcoholic drinks that you had in a 24-hour period?

☐ 12 or more drinks
☐ 9 to 11 drinks
☐ 7 to 8 drinks
☐ 5 to 6 drinks
☐ 3 to 4 drinks
☐ 1 to 2 drinks

14. Thinking back over your lifetime, was there a period when you drank more alcohol than you have in the last 12 months?

☐ Yes
☐ No (go to question 18)

15. What age were you during this period when you drank more alcohol? (tick all that apply)

☐ Under 20 years old
☐ 21 to 25 years old
☐ 26 to 30 years old
☐ Over 31 years old

16. During the period when you drank more alcohol, on average, how often did you have an alcoholic drink?

☐ Daily
☐ 3 to 6 times a week
☐ 1 to 2 times a week
☐ 2 to 3 times a month
☐ Less than once a month

17. During the period when you drank more alcohol, how many drinks did you have on a typical day when you drank alcohol?
Note: a drink is defined as one can of beer (330 mL), one glass of wine (100 mL), or 30 mL of straight spirits.
- ☐ 12 or more drinks
- ☐ 9 to 11 drinks
- ☐ 7 to 8 drinks
- ☐ 5 to 6 drinks
- ☐ 3 to 4 drinks
- ☐ 1 to 2 drinks

18. Do you smoke cigarettes regularly? (that is one or more a day)
Note: by cigarette we mean any tobacco product including roll your owns (with or without a filter), pipes, or chewing tobacco.
- ☐ Yes
- ☐ No
- ☐

19. Have you ever been a regular smoker of one or more cigarettes a day?
- ☐ Yes
- ☐ No (go to question 22)

20. If you are a current or previous regular smoker, how many years have you / did you smoke for?
- ☐ Greater than 20 years
- ☐ 11 to 20 years
- ☐ 5 to 10 years
- ☐ 2 to 4 years
- ☐ Less than 2 years

21. If you are a current or previous regular smoker, which statement best describes how many cigarettes per day you had?
- ☐ Greater than 21 cigarettes per day
- ☐ Between 16 and 20 cigarettes per day
- ☐ Between 11 and 15 cigarettes per day
- ☐ Between 6 and 10 cigarettes per day
- ☐ Between 4 and 5 cigarettes per day
- ☐ Between 2 and 3 cigarettes per day
- ☐ One cigarette per day

The following four questions deal with drug usage, remember ALL responses to this questionnaire are STRICTLY CONFIDENTIAL and available only to the researchers.

22. Have you ever used marijuana?
- ☐ Yes
- ☐ No (go to question 26)

23. If you are a current or previous user of marijuana, how many years have you / did you use for?
- ☐ Greater than 20 years
- ☐ 11 to 20 years
- ☐ 5 to 10 years

☐ 2 to 4 years
☐ Less than 2 years
☐ I have used marijuana less than 5 times in my life (go to question 26)

24. If you are a current or previous user of marijuana, how often did you/ do you use marijuana?
☐ A few times a year
☐ Once or twice a month
☐ Once or twice a week
☐ Almost every day

25. If you are a current or previous user of marijuana, how much money did/do you spend on marijuana in an average week?
☐ $1 to $20
☐ $21 to $40
☐ $41 to $60
☐ Over $61

<u>Section C: Sexual behaviours and sexual health</u>

26. At what age did you first have sexual intercourse? (this excludes oral sex)
Note: sexual intercourse includes both vaginal and anal sex.
☐ 15 years old, or younger
☐ 16 to 19 years old
☐ 20 years old, or older
☐ I have never had sexual intercourse (go to question 29)

27. What is your lifetime number of sexual intercourse partners? (this excludes oral sex)
☐ 1 or 2 partners
☐ 3 to 5 partners
☐ 6 to 10 partners
☐ 11 to 20 partners
☐ 21 to 49 partners
☐ Greater than 50 partners

28. What is your total number of sexual intercourse partners before age 25? (this excludes oral sex)
☐ 1 or 2 partners
☐ 3 to 5 partners
☐ 6 to 10 partners
☐ 11 to 20 partners
☐ 21 to 49 partners
☐ Greater than 50 partners

29. At what age did you first give/perform oral sex?
☐ 15 years old, or younger
☐ Between 16 and 19 years old
☐ 20 years old, or older
☐ I have never had oral sex (go to question 32)

30. What is your lifetime number of partners you have given oral sex to?

- ☐ 1 or 2 partners
- ☐ 3 to 5 partners
- ☐ 6 to 10 partners
- ☐ 11 to 20 partners
- ☐ 21 to 49 partners
- ☐ Greater than 50 partners

31. What is your total number of partners you have given oral sex to before age 25?

- ☐ 1 or 2 partners
- ☐ 3 to 5 partners
- ☐ 6 to 10 partners
- ☐ 11 to 20 partners
- ☐ 21 to 49 partners
- ☐ Greater than 50 partners

32. Which statement best describes your condom use before age 25?

Note: this question refers to condom use for vaginal, anal, and oral sex

- ☐ I never used condoms
- ☐ I rarely used condoms
- ☐ I sometimes used condoms
- ☐ I usually used condoms
- ☐ I always used condoms

33. Have you ever had a sexually transmitted infection/s? (such as Chlamydia, Gonorrhoea, Herpes or genital warts)

- ☐ Yes
- ☐ No

34. Has a sexual partner ever had a sexually transmitted infection/s? (such as Chlamydia, Gonorrhoea, Herpes or genital warts)

- ☐ Yes
- ☐ No
- ☐ Unsure

Males only please go to Section D: medical history and medication use (question 38)
Females answer question 35 - 37 before going to Section D

35. Have you had a cervical smear test in the past five years?

- ☐ Yes
- ☐ No
- ☐ Unsure

36. Have you ever had an abnormal cervical smear?

- ☐ Yes
- ☐ No (go to question 38)
- ☐ Unsure

37. What age were you when you had your abnormal smear/s? (tick all that apply)

- ☐ Under 25 years old
- ☐ 26 to 35 years old
- ☐ 36 years old, or older

<u>Section D: Medical history and medication use</u>

38. Have you ever had, or currently suffer from any of the following medical conditions? (tick all that apply)

- ☐ Tonsillitis
- ☐ Glandular fever
- ☐ Gastric Reflux
- ☐ Childhood papilloma of the voice box
- ☐ Type 1 (insulin dependent) diabetes
- ☐ Type 2 (adult onset / non-insulin dependent) diabetes
- ☐ HIV / AIDS
- ☐ Rheumatoid arthritis
- ☐ Early onset rheumatoid arthritis / juvenile idiopathic arthritis
- ☐ Asthma (requiring an inhaler)
- ☐ An autoimmune disease (such as multiple sclerosis (MS), lupus, or Crohn's disease)
- ☐ Heart disease (such as coronary artery disease, angina, arrhythmia, or heart failure)
- ☐ Rheumatic fever
- ☐ Other significant medical condition (please specify)____

39. Have you had your tonsils removed? (prior to your current diagnosis)

- ☐ No
- ☐ Yes (if yes please state when they were removed – if unsure give a timeframe ie 'during childhood' or 'greater than ten years ago' etc)

  ____

40. Have you ever had any of the following in / around your throat area (before your current biopsy, (tick all that apply))?

- ☐ Severe injury or trauma
- ☐ Surgery
- ☐ Chemotherapy or radiation

41. Have you ever had an organ transplant?

- ☐ No (go to question 43)
- ☐ Yes (if yes please state what organ and the year of the transplant)

  ____

42. If you have had an organ transplant, are you on immunosuppressive therapy?

- ☐ No
- ☐ Yes (if yes please state which therapy you are on)

  ____

43. During the last 12 months how often did you take aspirin?

- ☐ Daily
- ☐ 4 to 6 times a week
- ☐ 2 to 3 times a week
- ☐ Once a week
- ☐ Less than once a week
- ☐ I have not taken aspirin in the last 12 months

44. **During the last 12 months, how often did you take anti-inflammatory drugs (NSAID's) other than aspirin?**
(These are drugs for pain and inflammation relief and include ibuprofen (eg. Nurofen) and Voltaren. NOTE Paracetamol is not an NSAID)
    - ☐ Daily
    - ☐ 4 to 6 times a week
    - ☐ 2 to 3 times a week
    - ☐ Once a week
    - ☐ Less than once a week
    - ☐ I have not taken any NSAID's in the last 12 months

45. **Thinking back over your lifetime, was there a period when you used more aspirin or anti-inflammatory drugs (NSAID's) than you have in the last 12 months?**
    - ☐ Yes
    - ☐ No (go to question 47)

46. **Thinking about this period when you used more aspirin or anti-inflammatory drugs (NSAID's) please answer the following:**
    - Drug used: aspirin / anti-inflammatory (NSAID) (circle drug used)
    - Duration of heavier use (e.g. one month, 6 months etc)

    ____
    - Average frequency during this period (e.g. Daily, twice weekly, etc)

    ____
    - Average dose at each use (e.g. 2 x 500 mg tablets)

    ____
    - How long ago was the period of heavier use?

    ____

47. **Have you ever been prescribed any steroid drugs?** (such as Prednisone or cortisone)
    - ☐ Yes
    - ☐ No (go to question 49)

48. **If you have been prescribed steroid drugs please state when they were prescribed and the amount of time you took them for**

    ____

    ____

49. **Have you ever, or do you currently suffer from any allergies?** (such as hayfever, eczema, and food or drug allergies)
    - ☐ No
    - ☐ Yes (please list below, and tell us when they were diagnosed and/or symptoms started)

    ____

    ____

50. In the six months prior to your biopsy did you experience any of the following?

- ☐ Difficulty or pain in swallowing
- ☐ Difficulty in opening your mouth and/or jaw
- ☐ Hoarseness of your voice
- ☐ Non-healing lesions of the mouth
- ☐ Unusual bleeding in your mouth and/or throat
- ☐ A feeling of something being ‘stuck’ in your throat
- ☐ Earache
- ☐ A persistent sore throat (i.e. one that didn’t go away after antibiotics)
- ☐ An unusual smelling body odour / sweat
- ☐ Notice a lump/s in your neck
- ☐ Other (please specify) ____

If you have any comments or information you feel may be useful for the project please tell us here

____

____

____

____

____

____

Thank you for taking the time to complete this questionnaire,
Please return the questionnaire using the envelope provided.

Appendix 13 – Questionnaire for retrospective patients

Human Papillomavirus (HPV) associated oropharyngeal carcinoma

Participant Questionnaire

All responses to this questionnaire are STRICTLY CONFIDENTIAL and available only to the researchers.

Nothing that could identify you will ever be used when the results of this study are published.

- **The purpose of this questionnaire is to find out more about the risk factors for HPV positive oropharyngeal cancer. Many questions are based on risk factors reported by overseas studies.**
- **You may think some of the questions ask for sensitive information. It is your choice whether you choose to answer any question in the questionnaire.**
- **We understand some questions ask you to recall information from a long time ago, all the questions included will help improve our understanding of oropharyngeal cancer and we appreciate you taking the time to answer the questions as honestly as possible.**
- **There is a comments section at the end of the questionnaire, feel free to add in any extra information you feel may be useful for the study.**
- **If you have any queries about any part of this questionnaire please contact Rebecca Lucas-Roxburgh**
  - **Phone: 06 356 9099 extension 85797**
  - **Email:** R.Lucas-Roxburgh@massey.ac.nz

Section A: General information

51. Are you?

☐ Male
☐ Female

52. What age group where you when you were diagnosed with oropharyngeal cancer?

☐ 40 years or younger
☐ 41 – 45 years old
☐ 46 – 50 years old
☐ 51 – 55 years old
☐ 56 – 60 years old
☐ 61 – 65 years old
☐ 66 – 70 years old
☐ 71 years or older

53. Which one of these statements best describes your marital status?

- ☐ Single
- ☐ I am married / in a civil union / with a de facto partner
- ☐ In a relationship

54. Which ethnic groups do you belong to? (tick all that apply)

- ☐ New Zealand European
- ☐ Other European
- ☐ Maori
- ☐ Samoan
- ☐ Cook Island Maori
- ☐ Tongan
- ☐ Niuean
- ☐ Chinese
- ☐ Indian
- ☐ Other (please state) ________________________

55. Which statement best describes your total yearly income (before tax) for the 12 months before your diagnosis? (tick all that apply)

- ☐ Zero income
- ☐ Up to $14,000
- ☐ From $14,001 to $48,000
- ☐ From $48,001 to $70,000
- ☐ $70,001 and over
- ☐ Received Pension (New Zealand superannuation, or equivalent overseas pension)
- ☐ Received work and income benefit (either job seeker support, sole parent support or supported living payment)

56. What is your highest qualification?

- ☐ None
- ☐ High school qualification (such as NCEA levels 1-3, school certificate, sixth form certificate, higher leaving certificate, university entrance)
- ☐ University qualification (degree, diploma, certificate)
- ☐ Postgraduate degree
- ☐ Trade Certificate
- ☐ Other (please state) ___________________________

57. What is your occupation?____

58. If you have had other occupations please list them here:

____

____

59. During your working life, have you been exposed to potentially harmful substances (eg. chemicals, fumes, gas, dust, solvents, acids, pesticides, asbestos)

☐ Yes
☐ No (go to question 11)

60. Please tell us more about what you were exposed to:

- Type of substance:____
- How long you were exposed for:____
- Was any safety equipment used (eg. Masks, gloves etc): ____________________

____

Section B: Alcohol use and smoking history

61. In the 12 months before your diagnosis, on average, how often did you have an alcoholic drink?
☐ Daily
☐ 3 to 6 times a week
☐ 1 to 2 times a week
☐ 2 to 3 times a month
☐ Less than once a month
☐ I do not drink alcohol (go to question 14)

62. In the 12 months before your diagnosis, how many alcoholic drinks did you have on a typical day when you drank alcohol?
Note: a drink is defined as one can of beer (330 mL), one glass of wine (100 mL), or 30 mL of straight spirits.
☐ 12 or more drinks
☐ 9 to 11 drinks
☐ 7 to 8 drinks
☐ 5 to 6 drinks
☐ 3 to 4 drinks
☐ 1 to 2 drinks

63. In the 12 months before your diagnosis, what was the largest number of alcoholic drinks that you had in a 24-hour period?
☐ 12 or more drinks
☐ 9 to 11 drinks
☐ 7 to 8 drinks
☐ 5 to 6 drinks
☐ 3 to 4 drinks
☐ 1 to 2 drinks

64. Thinking back over your lifetime, was there a period when you drank more alcohol than you did in the 12 months before your diagnosis?
☐ Yes
☐ No (go to question 18)

65. What age were you during this period when you drank more alcohol? (tick all that apply)
☐ Under 20 years old

☐ 21 to 25 years old
☐ 26 to 30 years old
☐ Over 31 years old

66. During the period when you drank more alcohol, on average, how often did you have an alcoholic drink?

☐ Daily
☐ 3 to 6 times a week
☐ 1 to 2 times a week
☐ 2 to 3 times a month
☐ Less than once a month

67. During the period when you drank more alcohol, how many drinks did you have on a typical day when you drank alcohol?

Note: a drink is defined as one can of beer (330 mL), one glass of wine (100 mL), or 30 mL of straight spirits.

☐ 12 or more drinks
☐ 9 to 11 drinks
☐ 7 to 8 drinks
☐ 5 to 6 drinks
☐ 3 to 4 drinks
☐ 1 to 2 drinks

68. Before your diagnosis, did you smoke cigarettes regularly? (that is one or more a day)

Note: by cigarette we mean any tobacco product including roll your owns (with or without a filter), pipes, or chewing tobacco.

☐ Yes
☐ No

69. Have you ever been a regular smoker of one or more cigarettes a day?

☐ Yes
☐ No (go to question 22)

70. If you are a current or previous regular smoker, how many years have you / did you smoke for?

☐ Greater than 20 years
☐ 11 to 20 years
☐ 5 to 10 years
☐ 2 to 4 years
☐ Less than 2 years

71. If you are a current or previous regular smoker, which statement best describes how many cigarettes per day you had?

☐ Greater than 21 cigarettes per day
☐ Between 16 and 20 cigarettes per day
☐ Between 11 and 15 cigarettes per day
☐ Between 6 and 10 cigarettes per day
☐ Between 4 and 5 cigarettes per day
☐ Between 2 and 3 cigarettes per day
☐ One cigarette per day

<u>The following four questions deal with drug usage, remember ALL responses to this questionnaire are STRICTLY CONFIDENTIAL and available only to the researchers.</u>

72. Have you ever used marijuana?
- ☐ Yes
- ☐ No (go to question 26)

73. If you are a current or previous user of marijuana, how many years have you / did you use for?
- ☐ Greater than 20 years
- ☐ 11 to 20 years
- ☐ 5 to 10 years
- ☐ 2 to 4 years
- ☐ Less than 2 years
- ☐ I have used marijuana less than 5 times in my life (go to question 26)

74. If you are a current or previous user of marijuana, how often did you/ do you use marijuana?
- ☐ A few times a year
- ☐ Once or twice a month
- ☐ Once or twice a week
- ☐ Almost every day

75. If you are a current or previous user of marijuana, how much money did/do you spend on marijuana in an average week?
- ☐ $1 to $20
- ☐ $21 to $40
- ☐ $41 to $60
- ☐ Over $61

<u>Section C: Sexual behaviours and sexual health</u>

76. At what age did you first have sexual intercourse? (this excludes oral sex)
Note: sexual intercourse includes both vaginal and anal sex.
- ☐ 15 years old, or younger
- ☐ 16 to 19 years old
- ☐ 20 years old, or older
- ☐ I have never had sexual intercourse (go to question 29)

77. What is your lifetime number of sexual intercourse partners? (this excludes oral sex)
- ☐ 1 or 2 partners
- ☐ 3 to 5 partners
- ☐ 6 to 10 partners
- ☐ 11 to 20 partners
- ☐ 21 to 49 partners
- ☐ Greater than 50 partners

78. What is your total number of sexual intercourse partners before age 25? (this excludes oral sex)

☐ 1 or 2 partners
☐ 3 to 5 partners
☐ 6 to 10 partners
☐ 11 to 20 partners
☐ 21 to 49 partners
☐ Greater than 50 partners

79. At what age did you first give/perform oral sex?

☐ 15 years old, or younger
☐ Between 16 and 19 years old
☐ 20 years old, or older
☐ I have never had oral sex (go to question 32)

80. What is your lifetime number of partners you have given oral sex to?

☐ 1 or 2 partners
☐ 3 to 5 partners
☐ 6 to 10 partners
☐ 11 to 20 partners
☐ 21 to 49 partners
☐ Greater than 50 partners

81. What is your total number of partners you have given oral sex to before age 25?

☐ 1 or 2 partners
☐ 3 to 5 partners
☐ 6 to 10 partners
☐ 11 to 20 partners
☐ 21 to 49 partners
☐ Greater than 50 partners

82. Which statement best describes your condom use before age 25?
Note: this question refers to condom use for vaginal, anal, and oral sex

☐ I never used condoms
☐ I rarely used condoms
☐ I sometimes used condoms
☐ I usually used condoms
☐ I always used condoms

83. Have you ever had a sexually transmitted infection/s? (such as Chlamydia, Gonorrhoea, Herpes or genital warts)

☐ Yes
☐ No

84. Has a sexual partner ever had a sexually transmitted infection/s? (such as Chlamydia, Gonorrhoea, Herpes or genital warts)

☐ Yes
☐ No
☐ Unsure

Males only please go to Section D: medical history and medication use (question 38)

Females answer question 35 - 37 before going to Section D

85. Did you have a cervical smear test in the five years leading up to your diagnosis?

- ☐ Yes
- ☐ No
- ☐ Unsure

86. Have you ever had an abnormal cervical smear?

- ☐ Yes
- ☐ No (go to question 38)
- ☐ Unsure

87. What age were you when you had your abnormal smear/s? (tick all that apply)

- ☐ Under 25 years old
- ☐ 26 to 35 years old
- ☐ 36 years old, or older

Section D: Medical history and medication use

88. Have you ever had any of the following medical conditions? (tick all that apply)

- ☐ Tonsillitis
- ☐ Glandular fever
- ☐ Gastric Reflux
- ☐ Childhood papilloma of the voice box
- ☐ Type 1 (insulin dependent) diabetes
- ☐ Type 2 (adult onset / non-insulin dependent) diabetes
- ☐ HIV / AIDS
- ☐ Rheumatoid arthritis
- ☐ Early onset rheumatoid arthritis / juvenile idiopathic arthritis
- ☐ Asthma (requiring an inhaler)
- ☐ An autoimmune disease (such as multiple sclerosis (MS), lupus, or Crohn's disease)
- ☐ Heart disease (such as coronary artery disease, angina, arrhythmia, or heart failure)
- ☐ Rheumatic fever
- ☐ Other significant medical condition (please specify) ____

89. Have you had your tonsils removed? (prior to your diagnosis)

- ☐ No
- ☐ Yes (if yes please state when they were removed – if unsure give a timeframe ie 'during childhood' or 'greater than ten years ago' etc)

  ____

90. **Have you ever had any of the following in / around your throat area** (before your diagnosis (tick all that apply))?
    - ☐ Severe injury or trauma
    - ☐ Surgery
    - ☐ Chemotherapy or radiation

91. **Have you ever had an organ transplant?**
    - ☐ No (go to question 43)
    - ☐ Yes (if yes please state what organ and the year of the transplant)

    ____

92. **If you have had an organ transplant, are you on immunosuppressive therapy?**
    - ☐ No
    - ☐ Yes (if yes please state which therapy you are on)

    ____

93. **In the 12 months before your diagnosis, how often did you take aspirin?**
    - ☐ Daily
    - ☐ 4 to 6 times a week
    - ☐ 2 to 3 times a week
    - ☐ Once a week
    - ☐ Less than once a week
    - ☐ I have not taken aspirin in the last 12 months

94. **In the 12 months before your diagnosis, how often did you take anti-inflammatory drugs (NSAID's) other than aspirin?**
    (These are drugs for pain and inflammation relief and include ibuprofen (eg. Nurofen) and Voltaren. NOTE Paracetamol is not an NSAID)
    - ☐ Daily
    - ☐ 4 to 6 times a week
    - ☐ 2 to 3 times a week
    - ☐ Once a week
    - ☐ Less than once a week
    - ☐ I have not taken any NSAID's in the last 12 months

95. **Thinking back over your lifetime, was there a period when you used more aspirin or anti-inflammatory drugs (NSAID's) than you did in the 12 months before your diagnosis?**
    - ☐ Yes
    - ☐ No (go to question 47)

96. **Thinking about this period when you used more aspirin or anti-inflammatory drugs (NSAID's) please answer the following:**
    - Drug used: aspirin / anti-inflammatory (NSAID) (circle drug used)
    - Duration of heavier use (e.g. one month, 6 months etc)

      ____
    - Average frequency during this period (e.g. Daily, twice weekly, etc)

      ____
    - Average dose at each use (e.g. 2 x 500 mg tablets)

      ____
    - How long ago was the period of heavier use?

      ____

97. Have you ever been prescribed any steroid drugs? (such as Prednisone or cortisone)

☐ Yes
☐ No (go to question 49)

98. If you have been prescribed steroid drugs please state when they were prescribed and the amount of time you took them for

____

____

99. Have you ever, or do you currently suffer from any allergies? (such as hayfever, eczema, and food or drug allergies)

☐ No
☐ Yes (please list below, and tell us when they were diagnosed and/or symptoms started)

____

____

100. In the six months prior to your diagnosis did you experience any of the following?

☐ Difficulty or pain in swallowing
☐ Difficulty in opening your mouth and/or jaw
☐ Hoarseness of your voice
☐ Non-healing lesions of the mouth
☐ Unusual bleeding in your mouth and/or throat
☐ A feeling of something being 'stuck' in your throat
☐ Earache
☐ A persistent sore throat (i.e. one that didn't go away after antibiotics)
☐ An unusual smelling body odour / sweat
☐ Notice a lump/s in your neck
☐ Other (please specify) ____

If you have any comments or information you feel may be useful for the project please tell us here

____

____

____

____

____

Thank you for taking the time to complete this questionnaire,
Please return the questionnaire using the envelope provided.

Appendix 14 – Chapter four data frequency tables

Table 1: Demographic characteristics of study population by p16 status.

Variable	Level	p16 positive	p16 negative
Gender	Male	63	4
	Female	15	4
Age Group at diagnosis	40 or younger	2	0
	41 – 45 years old	4	0
	46 – 50 years old	5	0
	51 – 55 years old	20	1
	56 – 60 years old	11	1
	61 – 65 years old	18	0
	66 – 70 years old	8	2
	71 years or older	11	4
Current marital status	Single	17	3
	I am married /civil union /de facto partner	54	5
	In a relationship	8	0
Ethnicity	New Zealand European	67	8
	Other European	9	0
	Maori	5	0
	Samoan	0	0
	Cook Island Maori	0	0
	Tongan	1	0
	Niuean	1	0
	Chinese	0	0
	Indian	0	0
	Other	5	0
Income	Zero income	2	1
	Up to $14,000	4	0
	From $14,001 to $48,000	17	3
	From $48,001 to $70,000	20	0
	$70,001 and over	22	0
	Received Pension	10	4
	Received work and income benefit	1	0
Highest qualification	None	16	2
	High school qualification	18	2
	University qualification	21	2
	Postgraduate degree	5	1
	Trade Certificate	16	1
	Other	2	0

Table 2: Current occupation industry by Australian and New Zealand Standard Industrial Classification (***ANZSIC***).

NZIOC category	p16 positive	p16 negative
Agriculture, forestry and fishing	3	0
Manufacturing	3	0
Construction	7	0
Wholesale trade	2	0
Retail trade and accommodation	1	0
Transport, postal and warehousing	7	0
Financial and Insurance services	1	0
Rental, hiring and real estate services	1	0
Professional, Scientific, Technical, Administrative and Support Services	9	0
Public Administration and Safety	3	0
Education and Training	3	0
Health Care and Social Assistance	5	1
Retired	20	4
Response not classifiable	8	0
No response	6	2

Table 3: Current and lifetime alcohol use of study participants by p16 status.

Variable	Level	p16 positive	p16 negative
How often did you drink	Daily	12	1
	3 to 6 times a week	22	2
	1 to 2 times a week	25	1
	2 to 3 times a month	4	1
	Less than once a month	11	1
	I do not drink alcohol	5	2
How many drinks	12 or more drinks	1	0
	9 to 11 drinks	3	0
	7 to 8 drinks	2	0
	5 to 6 drinks	4	1
	3 to 4 drinks	24	1
	1 to 2 drinks	40	4
Maximum number of drinks	12 or more drinks	10	0
	9 to 11 drinks	8	0
	7 to 8 drinks	13	0
	5 to 6 drinks	15	1
	3 to 4 drinks	14	3
	1 to 2 drinks	14	2
Period of more drinking	Yes	60	7
	No	19	1
What age during heavier drinking	Under 20 years old	22	2
	21 to 25 years old	35	4
	26 to 30 years old	27	2
	Over 31 years old	21	2
How often during heavier drinking	Daily	16	3
	3 to 6 times a week	24	0
	1 to 2 times a week	19	2
	2 to 3 times a month	0	1
	Less than once a month	1	0
How many drinks during heavier drinking	12 or more drinks	6	0
	9 to 11 drinks	3	0
	7 to 8 drinks	9	2
	5 to 6 drinks	16	2
	3 to 4 drinks	20	2
	1 to 2 drinks	5	1

Table 4: Current and lifetime smoking history of study participants by p16 status.

Variable	Level	p16 positive	p16 negative
Current smoker	Yes	14	2
	No	65	6
Ever smoked	Yes	40	6
	No	38	2
How long for	Greater than 20 years	20	4
	11 to 20 years	7	1
	5 to 10 years	5	1
	2 to 4 years	5	0
	Less than 2 years	2	0
Cigarettes per day	Greater than 21 cigarettes per day	7	1
	Between 16 and 20 cigarettes per day	7	0
	Between 11 and 15 cigarettes per day	7	3
	Between 6 and 10 cigarettes per day	8	0
	Between 4 and 5 cigarettes per day	8	2
	Between 2 and 3 cigarettes per day	2	0
	One cigarette per day	0	0

Table 5: Lifetime marijuana use of study participants by p16 status.

Variable	Level	p16 positive	p16 negative
Ever used marijuana	Yes	46	0
	No	33	8
How long for	Greater than 20 years	5	0
	11 to 20 years	4	0
	5 to 10 years	5	0
	2 to 4 years	6	0
	Less than 2 years	5	0
	less than 5 times in my life	19	0
How often	A few times a year	7	0
	Once or twice a month	7	0
	Once or twice a week	10	0
	Almost every day	1	0
Spend per week	$1 to $20	17	0
	$21 to $40	3	0
	$41 to $60	1	0
	Over $61	0	0

Table 6: Number of oral and penetrative sex partners by p16 status.

Variable	Level	p16 positive	p16 negative
Age at debut	15 years old, or younger	11	2
	16 to 19 years old	58	2
	20 years old, or older	10	3
	I have never had sexual intercourse	0	0
Lifetime number of partners	1 or 2 partners	6	2
	3 to 5 partners	15	5
	6 to 10 partners	16	0
	11 to 20 partners	22	1
	21 to 49 partners	11	0
	Greater than 50 partners	8	0
Partners before age 25	1 or 2 partners	19	3
	3 to 5 partners	23	1
	6 to 10 partners	21	0
	11 to 20 partners	9	1
	21 to 49 partners	4	0
	Greater than 50 partners	2	0
Oral sex age at debut	15 years old, or younger	5	0
	16 to 19 years old	29	1
	20 years old, or older	39	1
	I have never had oral sex	5	4
Oral sex lifetime partners	1 or 2 partners	20	1
	3 to 5 partners	17	1
	6 to 10 partners	16	0
	11 to 20 partners	12	0
	21 to 49 partners	1	0
	Greater than 50 partners	6	0
Oral sex partners before age 25	1 or 2 partners	35	1
	3 to 5 partners	19	1
	6 to 10 partners	8	0
	11 to 20 partners	2	0
	21 to 49 partners	2	0
	Greater than 50 partners	2	0

Table 7: Sexual health information by p16 status

Variable	Level	p16 positive	p16 negative
Condom use before age 25	I never used condoms	23	4
	I rarely used condoms	27	0
	I sometimes used condoms	19	2
	I usually used condoms	9	1
	I always used condoms	1	0
Ever had STI	Yes	32	1
	No	47	6
Partner ever had STI	Yes	31	0
	No	33	6
	Unsure	14	1
Had cervical smear in last 5 years	Yes	11	3
	No	4	1
	Unsure	0	0
Had an abnormal cervical smear	Yes	5	0
	No	10	4
	Unsure	0	0
Age at time of abnormal smear	Under 25 years old	1	0
	26 to 35 years old	4	0
	36 years old, or older	0	0

Table 8: Medical conditions and medical history by p16 status.

Variable	Level	p16 positive	p16 negative
Suffered from a medical condition	Tonsillitis	43	3
	Glandular fever	15	1
	Gastric Reflux	20	3
	Childhood papilloma of voice box	0	0
	Type 1 diabetes	1	0
	Type 2 diabetes	6	1
	HIV / AIDS	0	0
	Rheumatoid arthritis	3	0
	Juvenile idiopathic arthritis	0	0
	Asthma (requiring an inhaler)	9	2
	An autoimmune disease	1	0
	Heart disease	9	2
	Rheumatic fever	3	0
	Other significant medical condition	22	1
Had tonsils removed	Yes	23	3
	No	52	5
Had any in/around throat	Severe injury or trauma	1	1
	Surgery	6	1
	Chemotherapy or radiation	3	1
Had an organ transplant	Yes	1	0
	No	0	0
If transplant, on immunosuppressive therapy	Yes	0	0
	No	1	0

Table 9: Aspirin, Non-steroidal anti-inflammatory drug (NSAID) and steroid drug use by p16 status.

Variable	Level	p16 positive	p16 negative
Aspirin use in last 12 months	Daily	6	2
	4 to 6 times a week	0	0
	2 to 3 times a week	1	1
	Once a week	0	0
	Less than once a week	19	0
	Not taken aspirin in the last 12 months	52	4
NSAID use in last 12 months	Daily	6	0
	4 to 6 times a week	0	0
	2 to 3 times a week	3	0
	Once a week	5	1
	Less than once a week	34	2
	Not taken NSAID in the last 12 months	27	4
Period of heavier use	Yes	24	2
	No	53	6
Drug used during heavier use	Aspirin	5	0
	NSAID	21	2
Prescribed steroid drugs	Yes	26	4
	No	52	3

Table 10: presence and type of allergies suffered by p16 status.

Variable	Level	p16 positive	p16 negative
Ever suffered	Yes	24	3
	No	55	4
Type of allergy	Food	6	0
	Drug	3	1
	Hayfever	12	2
	Eczema	5	2

Table 11: Symptoms experienced in the six months before diagnosis by p16 status.

Symptom	p16 positive	p16 negative
Difficulty or pain in swallowing	10	5
Difficulty in opening mouth and/or jaw	3	1
Hoarseness of the voice	8	5
Non-healing lesions of the mouth	4	0
Unusual bleeding in the mouth and/or throat	2	0
A feeling of something being ‘stuck’ in the throat	14	3
Earache	14	2
A persistent sore throat	16	4
An unusual smelling body odour / sweat	2	1
Notice a lump/s in the neck	38	4
Other	17	1

Appendix 15 – free text comments

In the 4 years prior to my diagnosis I used an alcohol based mouthwash twice a day on most days. I now am aware that this may be linked to oral cancer. Sometimes the mouthwash had a whitening chemical included within it.
when I was 40 years an ayurvedic instructor told me I had a blockage in my throat chakra (neck)
hoarseness of the voice was not unusual as I was and had been smoking up to the time of diagnosis. Sporadically I felt something was stuck in my throat but the only 'noticable' indication of something being wrong was the large lump in my neck.
I noticed my tonsil being larger in 2012 went to gp. Prescribed abx Up untill I self reffered myself to ent specialists, I saw multiple gp in nz and Australia who dismissed it as a Diseases tonsil due to chronic tonsititis and would be prescribed abx on all visits. Perhaps had of being reffered earlier it would not have come to treatment? Always looked fleshly and a wart like appearance, used to show my non medical friends who even commented that it looked strange. It nearly reached the middle of my uvula.
the doctor thought I had a blocked saliva gland but it was not sore. 4 months later tests were done.
hope it helps with your research
I am sorry not more precise with dates. I was abused as a child and have no clue when it started just was always there in my memory and life.
I enjoy perfect health and have had no issues or problems since having 1 tonsil removed (cancerous) in 2003. I now drink very little but like a cigarette about twice a day. No problems since spleen removed in 1982. now 71 and happy and healthy!!
mother died at 78 from cancer in uterus. I used marijuana from 26-50 then quit for three years. Resumed on an irregular basis socially, mainly weekends. Quit again 1 year ago. Have resumed vaping m.j. during treatment to alleviate the pain and was able to reduce prescription drugs and constipation, completely cut out morphine and pain almost disappeared. Apologies for not doing this questionnaire sooner, if you need any more info please contact me again.
re sexual activity not something that one normally keeps a record of
re alcohol and smoking questions my only experience of smoking was in my teens and experimental only of brief durations probably haven't had more than 20 cigarettes in my life. Similarly with drinking never had a regular habit only occasional I have been t total for over 40 years. 17 years in household with 2x heavy smokers inside. Birth until leaving home
two days prior to noticing the growth at the back of my throat, I had been to the dentist and had an injection on that side of my mouth.
on reflection after the fact (diagnosis), noticed an increased gag reflex when cleaning teeth. Felt unable to open mouth as extended as previous. Now after treatment (2 months) gag reflex seems back to normal
Cervix removed 1995 (full hysterectomy, including ovaries) I have had a couple of 'vault' smears. My lichen sclerosis has resulted in a total regression of the genital area ~ no labia or clitoris.
I have a sweet tooth and feel too much sugar contributed to the weakening of oral tissue. I remember inhaling something from garden compost while out gardening. I had a personal

shock - work related, 3 months prior to diagnosis of a 3 month old growth.
only had tonsilectomy to remove 'infected' tonsil
the lump on my neck came up over a 2 day period. I was admitted to hospital for tests. Cancer was not diagnosed for about 4-5 months when a biopsy was done during an operation to remove lump. Result - full radical neck dissection
go to your GP as soon as you see a small lump on your neck. I didn't.
dentist thought looked like ulcer, my (then)gp did not think serious and rejected hospital appointment as they said not significant (what rubbish). I could not trust a GP ever again; having knowledge of how the "system" is run, I went to a specialist (cancer) + was in hospital in 30 hours. If I had not done that I would not be here today – lucky me!!!! I believe that major stress factors triggered a basically healthy body thus tipping the balance between inactive prob HPV and active; on both occasions of major distress: A) breakdown of marriage+ B) the loss of my mother and sister within a 4 month period; I further add that I have thought for a long time of the "common" environment – ie very very similar of vagina + throat, seems that these specific environments are not only alike but are the location of the dev of HPV+ that enables (triggered) cancer development: there is no way these are not connected!! Gosh sorry guys - writing a bit messy! Far from senile – I do have a lot to say about this!!
until finding a lump in my neck I had not noticed/felt any different than usual. As a welder I am used to having a burning/sore throat occasionally due to different types of daily welding.
in the last 50 years I have been breathing in welding fumes from stainless steel, aluminium, and carbon steel. In the last 4-5 years 2 other people I have worked with have had throat cancer.
I did have a sore throat but it occurred the same time I felt the lump in my neck. The tumour had possibly been growing on my tonsil for 6 months but I never felt it.
Hi, this survey is fine but it needs to cover other aspectsincluding where one has worked, ehat substances one has been in contact with such as asbestos, fumes etc. just my opinion. For example I worked in several engineering shops, a foundry, Tiwai Aluminium smelter, a scrap yard etc where I was exposed to many substances. I also worked down the road from Ivan Watkins Dow in New Plymouth where agent orange was manufacturered. Thanks.
in the 2-3 years leading up to diagnosis I gradually ripped out and regibbed the rooms of my housecausing a lot of dust, including re-insulation. I rarely if ever wore a dust mask.
taking warfarin from 1996
I was under extreme stress at the time of diagnosis also when I was a retail butcher (age 30ish) a work colleague later developed throat cancer. He spoke of other workers in the same business with similar problems. Could this be too much exposure to flyspray. We certainly used a lot of it.
I worked with fibreglass for 4 years in the 60s, resins and acetone.
Earache symptoms were mild & the sore throat I originally thought was part of a cold I picked up at the end of June that persisted for about 7 weeks. When I investigated the soreness in my

throat I could not see anything using a mirror but when I stuck my finger near my tonsil I found there was a kind of lesion there. This 'lesion' was sensitive to the likes of fruit juice or even cold water. This pain went away as the lesion turned into a growth but I had already been back to the GP who said I had oral cancer. I'm glad it was initially painful else I might not have been diagnosed so soon as it would have been easy to ignore.
growth in throat was causing mild sleep apnea, but its presence was not recognised at the time.
the only thing that I noticed was a large tonsil and I choked one or twice. I never had a sore throat. Also there was a growing ball in my neck. This ball was noticed or started at 10mm 2 1/2 to 3 1/2 weeks later it was 30+ mm
I don't feel the questions relating to income and current employment status are relevant - very hard to remember prior symptoms from over 5 years ago sorry.
worked 8 hours a day breathing in heavy visible oil fumes for around 5 years in a dairy factory in Dargaville from 1965.

Appendix 16 – PISCF Brushings study

Participant Information Sheet

Your letterhead

Study title:	**Human papilloma virus (HPV) associated oropharyngeal carcinoma**		
Locality:	**Massey University, Palmerston North**	Ethics committee ref.:	**15/NTB/155**
Lead investigator:	**Rebecca Lucas-Roxburgh (MSc)**	Contact phone number:	**063569099 ext 85797**

You are invited to take part in a study on HPV in oropharyngeal cancer in New Zealand. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. We need your consent to be part of this study today, so we can collect an extra sample. However after your appointment feel free to talk about the study with other people, such as family, whānau, friends, or healthcare providers. If at any time after your appointment you decide you no longer want to be part of the study that is ok, and it is your right to change your mind. If you give consent today and change your mind later, just contact the researcher using the details at the end of this form and we will make sure your sample is not used in this study.

We are unable to provide interpreters for this study. All study material including the questionnaire is in English.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is 6 pages long, including the Consent Form. Please make sure you have read and understood all the pages.

WHAT IS THE PURPOSE OF THE STUDY?

This study aims to develop new tests for the early detection of HPV positive oropharyngeal cancer (OPC). The oropharynx is in the throat and OPC includes cancer of the oropharynx, tonsil, and base of the tongue. This study is important as new tests could lead to an earlier diagnosis, and may have a role in preventing HPV positive cancers. HPV is the cause of almost all cervical cancers. We now know that HPV causes a proportion of OPC. The incidence of HPV positive OPC has increased in many developed countries over the last two decades. The similarities between cervical and oropharyngeal cancer suggest that early detection and even prevention of OPC may be possible.

This project will apply new tests to recently collected oropharyngeal biopsy samples, and brushings of the tonsils. The project will also compare the risks for HPV positive versus HPV negative OPC using responses from a questionnaire.

Funding has been provided by Massey University, and the Hawkes Bay Medical Research Foundation. The lead investigator (Rebecca Lucas-Roxburgh) is a PhD student at Massey University. Dr's Howe and Benschop are the project supervisors and are Massey University staff. Dr Lockett is the third project supervisor and he is a pathologist at MedLab Central. Rebecca Lucas-Roxburgh is the contact person for this study and her phone and email details are listed at the end of this information sheet.

This study will commence in October 2015 and will take approximately two years. Health and Disabilities Commission Ethics Committee (HDEC) approval has been sought and granted for this study (HDEC reference: 15/NTB/155).

WHAT WILL MY PARTICIPATION IN THE STUDY INVOLVE?

You have been chosen to participate in this study because of your recent oropharyngeal cancer diagnosis. Participation in this study means when the lab has finished with your biopsy it will be sent to Massey University for use in this project. As well as your biopsy the surgeon will also take a brushing of your tonsils. This is done with a small brush that looks like a mascara brush.

Participation also involves you filling out a questionnaire. The questionnaire will be posted to you in the weeks following your appointment. The questionnaire is to be filled out in your own time, either online or on paper and returned in the envelope provided. Participating in this study will not involve any extra appointments over your usual visits with your surgeon.

The purpose of the questionnaire is to find out more about the risk factors for HPV positive oropharyngeal cancer. The questions are based on risk factors that have been reported by other international studies and include your alcohol intake, smoking, and medical history. HPV is a sexually transmitted virus and because of this the questionnaire contains a number of sensitive questions. It is your choice whether you choose to answer any question in the questionnaire. Your responses are confidential. Nothing that could identify you will ever be used when the results of this study are published.

What are the possible benefits and risks of this study?

Your surgeon will explain to you the process of taking a biopsy and any possible risks and side effects. They will also explain the brushing of your tonsils. This carries very little risk and is not invasive, but may be slightly uncomfortable.

The benefit of participating in this study is to improve our health knowledge. This study will develop new tests. These could lead to better outcomes for future patients and even have a role in cancer prevention.

Who pays for the study?

This study will be funded by various grants from Massey University and the Hawkes Bay Medical Research Foundation. You will not incur any costs by participating in this study.

What if something goes wrong?

If you were injured in this study, which is unlikely, you would be eligible for compensation from ACC just as you would be if you were injured in an accident at work or at home. You will have to lodge a claim with ACC, which may take some time to assess. If your claim is accepted, you will receive funding to assist in your recovery.

If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won't affect your cover.

What are my rights?

Participating in this study is your choice. You are free to decline to participate in this study, or withdraw from the research. If you give consent then at any time wish to withdraw please make contact with the lead investigator using the details at the end of this form.

As a participant you have the right to access any information that we collect about you. The type of information collected is described above in 'what will my participation in the study involve'.

Any information about you will only be accessed by the research team. We will use your information to get your specimen from the laboratory. After this we will only use a unique study number. Using a study number means once we have retrieved the specimens from the labs any of your personal information is de-identified. Hard copy information will be stored in a locked filing cabinet. Any digital information will be stored in a password protected computer file. No information that could identify you will ever be released as part of the findings of this research.

You may hold beliefs about a sacred and shared value of all or any tissue samples removed.

The cultural issues associated with sending your samples overseas and/or storing your tissue should be discussed with your family/whanau as appropriate. There are a range of views held by Maori around these issues; some iwi disagree with storage of samples

citing whakapapa and advise their people to consult prior to participation in research where this occurs. However it is acknowledged that individuals have the right to choose.

WHAT HAPPENS AFTER THE STUDY OR IF I CHANGE MY MIND?

It is your right to change your mind about participating in this study. We understand the explanation of the study and time to give consent at your appointment was limited. If you do decide later that you no longer want to be part of the study you can contact the researcher using the details at the end of this form. If you change your mind about participating we will send specimens back to the laboratory where they were processed, and will destroy the tonsillar brushing sample that was collected. If you change your mind after you have completed the questionnaire we will also destroy this and ensure none of your responses are used as part of the study.

Any data generated by this study will be stored in a de-identified form (ie. using only a study number not personal details) for at least five years so it can guide future research. The lead investigator is responsible for the secure storage of data. As mentioned above this involves the use of lockable filing cabinets and password protected computer files.

After completion of the study the biopsy samples used in this study will be returned to the laboratories where the diagnostic testing took place. These samples will then be stored as per standard laboratory protocols. The tonsillar brushings will be kept for five years to allow testing to be completed. After this time they will then be destroyed.

Once the study is complete results will be communicated at scientific conferences, and by publication in scientific journals. Participants are able to request a summary of the study's findings by ticking the box on in the attached consent form. It is estimated that this study will take around two years. The summary of results is therefore likely to be made available during 2018.

WHO DO I CONTACT FOR MORE INFORMATION OR IF I HAVE CONCERNS?

If you have any questions, concerns or complaints about the study at any stage, you can contact:

Rebecca Lucas-Roxburgh, PhD student

Phone: 06 356 9099 extension 85797

Email: R.Lucas-Roxburgh@massey.ac.nz

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

Phone: 0800 555 050
Fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

For Maori Heath support please contact :

Dr Maureen Holdaway, Deputy Director of Maori Health and Development, College of Health, Massey University

Phone: 06 356 9099 extension 85092

Email: M.A.Holdaway@massey.ac.nz

You can also contact the health and disability ethics committee (HDEC) that approved this study on:

Phone: 0800 4 ETHICS

Email: hdecs@moh.govt.nz

Consent Form

Your letterhead

I have read, or have had read to me, and I understand the Participant Information Sheet.	
I have been given sufficient time to consider whether or not to participate in this study.	
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	
I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	
I consent to the research staff collecting and processing my information, including information about my health.	
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	
I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	
I know who to contact if I have any questions about the study in general.	
I understand my responsibilities as a study participant.	
I wish to receive a summary of the results from the study.	Yes ☐ No ☐

Declaration by participant:

I hereby consent to take part in this study.

Participant’s name:

Signature: Date:

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant’s questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher’s name:

Signature: Date: