UTS UNIVERSITY OF TECHNOLOGY SYDNEY

The detection of human papillomavirus in head and neck cancers

by Fiona Therese Deutsch

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of
Associate Professor Nham Tran
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University of Technology Sydney
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Certificate of original authorship

I, Fiona Therese Deutsch, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences, Faculty of Engineering and IT at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.
This document has not been submitted for qualifications at another other academic institution.

This research is supported by the Australian Government Research Training Program.

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Date: 5/ 1/ 2023

I dedicate my doctoral thesis to my parents; Jacqueline Deutsch-Doleman, Bruce Doleman, and Harry Deutsch for their endless love, support, and encouragement, that transcends space, time, and life.

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Thesis format

The current thesis is presented as a Thesis by Compilation and is structured as a single manuscript comprising a combination of distinct chapters and published/ publishable papers. Where applicable, published papers presented in this thesis have been amended to reflect updated literature. This thesis has been formatted in accordance with the guidelines outlined by the University of Technology Sydney at the time of this submission (January 2023).

Statement & list of papers and conferences

This thesis originates from collected works featured in,

Publications:

Deutsch F., Khoury S., Elliott, M. & Tran, N., 'The application of salivary non-coding RNAs for the diagnosis of oral cancers', ***Head and Neck***, 2020.

Deutsch F., Regina Bullen, I., Nguyen K., Ngoc-Ha T., Elliott, M. & Tran, N. 'Current state of play for HPV-positive oropharyngeal cancers, ***Cancer Treatment Reviews***, 2022.

Deutsch, F., Sais, D., Elliott, M. & Tran, N. 'A multiplex quantitative PCR liquid biopsy to detect viable human papillomavirus 16 from saliva', ***BMC Cancer***, 2023. Under review.

Deutsch F., Pham DX., Hien ND., Nguyen TV & Tran N. 'Trends in head and neck cancer incidence in Ho Chi Minh City between 1996-2015', ***Head and Neck***, 2023. Under review.

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Deutsch, F., Sais, D. & Tran, N. Multiplex approach to detect HPV in the saliva of Head and Neck Cancer patients. ***Chris O'Brien Lifehouse Symposium***. 2019, Sydney Australia.

Deutsch, F., Sais, D. & Tran, N. Multiplex RT-qPCR to detect HPV16 oncogenes in head and neck cancer patients. ***New Horizons***. 2019, Sydney Australia.

Deutsch, F., Sais, D. & Tran, N. Multiplex approach to detect E6/E7 mRNA for the prognosis of head and neck cancer patients. ***Faculty of Science Transdisciplinary ECR Retreat (FoSTER)***. 2019, Sydney Australia. Awarded Best Poster Presentation.

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Deutsch, F., Sais, D. Elliott, M. & Tran, N. Novel multiplex approach to identify viral mRNA for the detection of HPV positive head and neck cancer using human saliva. ***Australian Society for Medical Research, National Scientific Conference.*** 2022, Sydney Australia. Awarded Best Student Oral Presentation.

Publication Declaration

Paper I

Title	Current state of play for HPV-positive oropharyngeal cancers
Authors	Fiona T Deutsch, Izabel R Bullen, Kevin Nguyen, Michael S Elliott, Nham T Tran
Status	Published (2022)
Journal	Cancer Treatment Reviews
Inclusion	This paper is presented in Chapter 1

Paper II

Title	Application of salivary non-coding microRNAs for the diagnosis of oral cancers
Authors	Fiona T Deutsch, Samantha J Khoury, John B Sunwoo, Michael S Elliott, Nham T Tran
Status	Published (2020)
Journal	Head and Neck
Inclusion	This paper is presented in Chapter 2

Paper III

Title	A multiplex quantitative PCR liquid biopsy to detect viable human papillomavirus 16 from saliva
Authors	Fiona T Deutsch, Dayna Sais, Meredith Hill, Michael S Elliott, Nham T Tran
Status	Under Review (2023)
Journal	BMC Cancer
Inclusion	This paper is presented in Chapter 3

Paper IV

Title	Trends in head and neck cancer incidence in Ho Chi Minh City between 1996-2015.
Authors	Fiona Deutsch, Dung X Pham, Nguyen Dinh Hien, Tuan, V. Nguyen, and Nham Tran.
Status	Under Review (2023)
Journal	Head and Neck
Inclusion	This paper is presented in Chapter 4

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Abbreviations

Abbreviation	Term
ACTB	Beta actin
AJCC	American Joint Committee on Cancer
ASIR	Age-standardised incidence rates
ASR	Age-standardised rates
cDNA	Complementary DNA
CFS	Cell-free supernatant
circRNA	Circular RNA
CO2	Carbon dioxide
COE	Conventional oral examination
Cq	Quantification cycle
Ct	Cycle threshold
ddPCR	Digital droplet PCR
DNA	Deoxyribonucleic acid
ENE	Extra nodal extension
FDA	Food and Drugs Administration, United States
FFPE	Formalin-fixed paraffin-embedded
GLOBOCAN	IARC Global Cancer Observatory
GSO	General statistics office
HGD	High grade dysplasia
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
ISH	In situ hybridisation
LGD	Low grade dysplasia
LMIC	Low- and middle-income countries
LOD	Limit of detection
miRNA	MicroRNA
N	Nodal

Abbreviation	Term
NCBD	National Cancer Data Base (USA)
ncRNA	Non-coding RNA
NSW	New South Wales
OLP	Oral lichen planus
OPC	Oropharyngeal cancer
OPMD	Oral potentially malignant disorder
OSCC	Oral squamous cell carcinoma
OVL	Oral verrucous leucoplakia
PCR	Polymerase chain reaction
PiRNA	Piwi-interacting RNAs
PMD	Potentially malignant disorder
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase - polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
TGA	Therapeutic Goods Administration, Australia
TNM	Classification of malignant tumours (T, size of primary tumour; N, describes nearby lymph nodes that are involved; M, metastasis)
TORS	Trans-oral robotic surgery
tRFs	Transfer RNA fragments
tRNA	Transfer RNA
TSCC	Tongue squamous cell carcinoma
UTR	Untranslated region
WHO	World Health Organisation

COVID-19 Impact Statement

The COVID-19 pandemic and subsequent lockdowns demonstrably disrupted this doctoral thesis. The inability to conduct fieldwork and face-to-face research, with restricted or complete restriction of access to facilities, laboratories, and hospitals, affected my ability to collect and analyse data for this research. As a laboratory heavy research project that relies on the obtaining of patient specimens for analysis, the pandemic led to the restructuring of original plans, aims and methodologies to ensure the high quality of this doctoral dissertation.

In addition, a substantial amount of work to collect specimens and process and analyse them had to be abandoned when COVID-19 restrictions were implemented in NSW. We were unable to fully transition this part of the research to an online only format due to the nature of the project and unfortunately, only proof-of-concept research was able to be undertaken in Chapter 3, which was less than originally intended. Additionally, Chapter 4 describes an epidemiological research study, which was one mitigation strategy implemented during the COVID-19 lockdowns to ensure high quality research could continue to be undertaken while at home.

Abstract

Head and neck cancer is the sixth most common cancer type worldwide, with 5-year survival rates for late-stage diagnosis less than 50%. Over the last ten years, there have been a significant increase in human papillomavirus (HPV) infections associated with head and neck cancers. Today, this sexually transmitted virus is the driver of cancer progression in cases of tongue and throat cancers, referred to as oropharyngeal cancers (OPC). More than 85% of these patients are infected with the high-risk HPV strain, HPV16.

Furthermore, there is increasing evidence that HPV-related OPCs constitute an epidemiological, molecular, and clinically distinct form, and therefore, the treatment of this specific subtype of OPCs should adopt a distinct clinical treatment pipeline. It is therefore imperative that OPCs are accurately tested for transcriptionally active viral HPV. Thus, the aim of this research was to develop a multiplex diagnostic assay to accurately detect HPV16 RNA oncogenes E6 and E7 using saliva from OPC patients in Sydney Australia.

For proof-of-concept, our lab's multiplex approach was able to detect E6 and E7 in HPV(+) cell lines with no significant difference in quantification of cycle (Cq) values or PCR efficiency to a traditional singleplex reaction. We then carried out serial dilutions of starting RNA input to determine the assay's limit of detection. Finally, we assessed our method in OPC patient tissue and saliva, which accurately differentiated between HPV-positive and negative specimens.

Due to COVID-19 restraints, the research pivoted into bioinformatics and epidemiology, looking into understanding how head and neck cancers are growing in Southeast Asia. With access to the Ho Chi Minh Cancer Registry, we were able to, for the first time, characterise head and neck cancer cases and its four main subtypes – oral cancer, oropharyngeal cancer, nasopharyngeal cancer, and laryngeal cancer in Vietnam between 1996 and 2015. Using a bioinformatics approach, this research sought to quantitate the incidence of head and neck cancer in Ho Chi Minh City, analyse age-standardised rates of head and neck cancer and examine defining points of incidence through joint point regression analysis.

Our analysis found that head and neck cancers are steadily growing in Vietnam, with oropharyngeal and oral cancers rising steeply. Of the four head and neck subtypes, Vietnamese people are affected by nasopharyngeal cancers more than the other subtypes, which corresponds to data in other SE Asian countries. The age-standardised rate (ASR) for this cancer type is also nearly

double that of global data. Interestingly, oropharyngeal ASR in Ho Chi Minh City matched global rates (1.1 per 100,000), which have increased quite significantly in the past decade due to the prevalence of HPV. With the coverage of the HPV vaccine in Vietnam traditionally low, our findings imply that a more aggressive screening strategy for identifying cancer cases earlier is warranted and the expansion of the coverage of the national vaccination target to males would be beneficial given the steady increase in oropharyngeal prevalence in Ho Chi Minh.

Overall, our research has demonstrated a non-invasive diagnostic assay to accurately detect HPV16 RNA oncogenes using saliva from OPC patients in Sydney Australia and has been the first study to characterise head and neck cancer cases in Ho Chi Minh City, Vietnam, as well as the cancer's notable subtypes.

Chapter 1

Current State of Play for HPV-positive Oropharyngeal Cancers

1.1 Copyright Information

This chapter has been published as an article in the journal of ***Cancer Treatment Reviews*:**
Cancer Treat Rev. 2022 Nov;110:102439. doi: 10.1016/j.ctrv.2022.102439. Epub 2022 Jul 26.

The text presented here is the accepted version of the manuscript. Numbering of sections, style of referencing, number of tables and figures are altered to align with the formatting of the thesis.

1.2 Author Contribution

Authors: Fiona T Deutsch, Izabel R Bullen, Kevin Nguyen, Michael S Elliott, Nham T Tran.

Fiona Deutsch (graduate student) is the first author of this Review article. Her contribution to this manuscript is demonstrated by the following roles/ tasks: researched the field, undertook a literature review, prepared the tables and figures, and was actively involved in the initial drafting, editing and review process.

Author permission to include this paper

I provide permission for Fiona Deutsch to include "Current state of play for HPV-positive oropharyngeal cancers" in her PhD thesis.

Signatures

Fiona Deutsch, MSc. Production Note: Signature removed prior to publication

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A/ Prof Nham T Tran, PhD. Production Note: Signature removed prior to publication

1.3 Abstract

Clinically, HPV-positive oropharyngeal cancers (OPCs) have been shown to have a distinct prognosis, compared to HPV-negative tumours, particularly in survival rates and responses to treatment. These patients have better survival chances and improved prognosis, indicating that a more exhaustive knowledge of these distinctions would aid in the discovery of clinical approaches for both HPV-positive and negative tumours. Furthermore, there is increasing evidence that HPV-related oropharyngeal cancers constitute an epidemiological, molecular, and clinical distinct form as compared to non-HPV related ones therefore, the treatment of these specific subtype of oropharyngeal cancers should adopt a distinct clinical treatment pipeline. Our review will examine the current approaches for the diagnosis and treatment of OPC and discuss the relevance of de-escalation clinical trials in progress.

1.4 Introduction

Head and neck squamous cell carcinomas (HNSCC) are a heterogenous group of cancers that affect the epithelium within the upper aero-digestive track [1]. Worldwide, these cancers can be attributed to more than five hundred thousand cases, ranking as the sixth most common malignancy [2]. The oropharynx, part of this tract, is a key site for oropharyngeal cancers (OPC) and includes structures such as the base of the tongue, soft palate, tonsils, and the pharyngeal walls. These cancers are distinct in their behavior and prognosis, often influenced by the specific anatomical subsite involved.

The complex aetiology of head and neck cancers comprises of both intrinsic and extrinsic factors. In addition to the two major risk factors associated with the development of head and neck cancer, particularly habitual tobacco and alcohol consumption, it is now broadly accepted that viral infections (e.g., Human Papillomavirus and the Epstein-Barr virus) are also critical in this cancer's pathogenesis [1]. Research has shown that chronic tobacco and alcohol consumption act synergistically in the development of head and neck cancer [2], whereas the HPV infection operates independently and has been implicated as a vital agent in the development of oropharyngeal cancers (OPC).

We now know that HPV induced OPC is a clinically distinct subset of HNSCC which is associated with overall better treatment outcomes and higher survival rates compared to HPV negative OPCs [4]. This suggests a distinct genomic and carcinogenic cascade which allows for the potential de-escalation of treatment which can reduce morbidities associated with OPC and increase quality of life. In order to successfully minimise acute and chronic toxicity in these patients, two crucial factors must be addressed: accuracy of identification of patients with HPV-related oropharyngeal squamous-cell carcinoma and of those with HPV-related carcinoma who would not benefit from de-escalation therapy. This paper discusses HPV's epidemiology in OPCs, examines the current approaches for the diagnosis and treatment of OPC and discusses the relevance of de-escalation clinical trials in progress.

1.5 HPV Epidemiology

Up to 70% of OPCs are HPV positive, with a large number being seen in the tonsils and base of tongue [5]. Moreover, 85% of OPCs are infected with a high-risk variant, such as HPV 16 or HPV 18 [6]. Recent research from the National Cancer Data Base (NCDB) study showed that HPV-positive rates were 62.9%. 17.7%, 11.0% and 10.6% in patients with oropharynx, hypopharynx, larynx, and oral cavity tumours, respectively.[7]

The International Agency for Research on Cancer (IARC) has classified 13 HPV types as group 1 carcinogens (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -66), which are commonly referred to as ‘‘high risk-HPVs’’ (hr-HPVs) [8, 9]. hr-HPV-16 further responds for most HPV-related cancers in other anogenital areas and the oropharynx [10]. Including HPV 16 and HPV 18, eight HPV types (alpha-7 types HPV 18 and 45, alpha-9 types 16, HPV 31, 33, 35, 52 and 58) were found as most common types in cancers in the IARC meta-analysis and the ICO study, in all regions of the world providing data [9].

The proportion of head and neck squamous cell carcinomas that are HPV-related is thought to be increasing, associated with a reduction in smoking rates and decreases in non-HPV driven squamous cell carcinoma (SCC) [11]. According to data provided by GLOBOCAN [12] and using global incidence rates of HPV using methodology from Plummer et al [13], we can see that virtually all cervical cancers are caused by HPV, while approximately 31% of oropharyngeal carcinomas are attributable to HPV (**see Table 1**). HPV incidence is also high in anal squamous cell carcinomas, vaginal carcinomas, and penal carcinomas with attributable rates of 88%, 78% and 51% respectively.

Not all subtypes in the head and neck area are affected equally by HPV; the tonsillar area appears to be particularly susceptible, with one study showing 70 per cent of tonsillar carcinomas to be HPV positive [14]. Interestingly, while high-risk HPV sequences were detected in oral cancer cells from 23 per cent of patients, these same variants were also detected in 11 per cent of control subjects [11]. This research therefore suggests that the role of hr-HPV in the oral cavity compared to the oropharynx may differ despite the presence of the high-risk genotype in both subtypes. Recent longitudinal research into the natural history of HPV in an Australian cohort has shown that HPV clearance rates were quite high, with 78% of the cohort clearing their oral HPV infection over 24 months [15]. The authors note that while most infections cleared in this period, HPV infections persisted in some which might predispose them to oropharyngeal carcinogenesis. This is consistent with clinical findings in cervical cancer, where many more patients are infected but do not develop early-stage cancers. It is estimated that 10 million women in the US have cervical human papillomavirus infections, while only 15 thousand develop cancer [11]. This suggests a more complex aetiology than previously thought, whereby the virus may lay dormant in the patient until an intrinsic or extrinsic factor (or factors) triggers the carcinogenic cascade.

Table 1. Estimated numbers of HPV-infection attributable cancer cases in 2020. Data was obtained from GLOBOCAN. HPV infection rates were calculated as described in Plummer et al 2016. HPV infection is attributable a majority of the cancer types analysed, with cervix uteri carcinomas being completely attributable to the virus and oral cancers the least likely to be caused by HPV infection.

	Males		Females		Total	
Cancer Types (ICD-11)	New cases	New cases attributable to infectious pathogens	New cases	New cases attributable to infectious pathogens	New cases	New cases attributable to infectious pathogens
Cervix uteri carcinoma (C53)			604,127	604,127	604,127	604,127
Oropharyngeal carcinoma (C09-10)	79,045	24,346	19,367	5,965	98,412	30,311
Oral cavity and lip carcinoma (C02-08)	265,211	11,404	113,502	4,881	377,713	16,242
Larynx carcinoma (C32)	160.27	7,372	24,350	1,120	184,615	8,492
Anus squamous cell carcinoma (C21)	21,706	19,101	29,159	25,660	50,865	44,761
Penis carcinoma (C60)	36,068	18,395			36,068	18,395
Vagina carcinoma (C52)			17,908	13,968.24	17,908	13,968.24
Vulva carcinoma (C51)			45,240	11,264.76	45,240	11,264.76

Abbreviations: ICD-11 refers to the 11th revision of the International Classification of Diseases.

The latest evaluation of the International Agency for Research in Cancer (IARC) on the carcinogenicity of HPV in patients concluded that (a) there is enough evidence for the carcinogenicity of HPV type 16 in the oral cavity, oropharynx (including tonsil cancer, base of tongue cancer and other oropharyngeal cancer sites), and (b) limited evidence for laryngeal cancer [8, 9]. There is increasing evidence that HPV-related oropharyngeal cancers constitute an epidemiological, molecular, and clinically distinct form as compared to non-HPV-related ones. Several studies indicate that head and neck cancers associated with HPV are sexually acquired oral HPV infections that were not cleared, persists, and evolves into neoplastic lesions [16, 17]. The most recent figures estimate that 25.6% of all oropharyngeal cancers are attributable to HPV infection with HPV16 being the most frequent type [18]. Overall, 2.2 million new cancer cases were attributable to infections in 2018, representing 13% of all cancer cases HPV for 690 000 new cases (ASIR 8.0 cases per 100 000 person-years), predominantly cervix uteri carcinoma [19]. Notably, oropharyngeal cancers caused by HPV are increasing in high-income countries, but their relative burden is still too small to affect the global trend due to cervical cancer [18].

1.6 Incidence of HPV-related oropharyngeal cancer by socioeconomic status

Squamous cell carcinoma of the oropharynx has increased in incidence in developed countries over the last 20 years [20-22]. HPV-related oropharyngeal cancer prevalence rates in these low middle-income countries range from 36% to greater than 80%, varying with geographical location and anatomical subsite [23-25]. It is likely that population-specific incidence rates of HPV-induced OPC are influenced by oral HPV infection rates, sexual behaviour, and rates of smoking and drinking. However, among HPV positive OPC, HPV16 is the predominant genotype, accounting for approximately 95% of cases [26].

The socioeconomic differences among OPC subgroups have implications for OPC prevention efforts, including tobacco cessation, behaviour modification and vaccination programs. According to the IARC, lip squamous cell carcinoma and oral squamous cell carcinomas are the fourth most common form of cancer and the sixth highest cause of death in LMICs [27]. However, despite this, few studies have given insight into the incidence of HPV-related OPC in lower socioeconomic countries and it should also be noted that the incidence of lip SCC in these LMICs are traditionally related to UV exposure [28]. Limited health system capacities and competing health priorities in low- and middle-income countries may explain why these cancers are grouped into oropharyngeal cancers, or head and neck cancers generally. Where research has been conducted in countries without effective coverage of screening, many people, especially those living in poverty, do not seek medical

attention until their disease is advanced, and no longer amenable to surgical intervention [29]. When these patients do seek care, treatment may often be unavailable, and financial protection is inadequate for most low-income families [29]. These factors contribute to the disproportionately high mortality from the disease in LMICs [29].

Where data has been collected in LMICs, it has shown that the majority of OPC patients are HPV-negative. For example, only 6.1% of OPCs from the largest Brazilian public cancer centre tested positive for HPV [30], compared to rates seen in the United States, where up to 70% of OPCs are HPV-related [31]. It should be noted however that the Brazilian study involved using a combination of qPCR for HPV16 E6 and p16 immunohistochemistry to identify HPV-positive cases. While this approach is robust, it may have contributed to the low detection rates as it confirms HPV presence only when both tests are positive, potentially missing cases where only one marker is positive. Interestingly, the Brazilian study also indicated that HPV-positivity did not impact on OPC patients' overall survival, which is in further contrast to the better overall survival rates seen in OPC patients in developed countries. This could be due to the high prevalence of tobacco and alcohol use among the Brazilian cohort, potentially overshadowing the prognostic benefit of HPV positivity. Recent research has also suggested the HPV positive smokers do not do much better than HPV negative smokers, with HPV positive non-smokers having the best prognosis [32]. This aetiology is also replicated in Asia, where the highest HPV- related head and neck cancers were found in South Asia (10.42%), followed by 5.8% in Southeast Asia and 5.7% in East Asia [33]. However, the exact prevalence of HPV associated and head and neck cancer among Asian populations is still debatable as medical records become more accurate and available.

In Latin America, information concerning HPV-related tumours aside from the uterine cervix are limited. A recent systematic review revealed not only high HPV prevalence at these sites, but also higher clearance rates of infection compared to the uterine cervix [34]. In the northeast region of Brazil and some areas of Argentina, anal cancer incidence rates reported are 0.2 to 1.4 per 100,000 persons/year [35, 36]. In Brazil, the study of Menezes et al, 2020, showed an emerging risk for HPV-related OPC/OCC in young people, HPV-related OPC/OCC in young males and females, increased by 3.8% and 8.6% per year, respectively [37]. More so, in 2012, Brazil had an age-standardised incidence rate of head and neck cancer cases attributable to HPV around 0.3% (Age−standardised incidence rate per 100,0000 women World Standard) [38].

1.7 The use of p16 surrogacy and the 8th Ed AJCC/ UICC TNM system for OPC staging

In 2017, the American Joint Committee on Cancer (AJCC) divided OSCC tumours in two distinct diseases, with specific TNM classifications for HPV-negative and HPV positive OSCC, and HPV positivity evaluated by p16 immunostaining as the minimal condition for establishing clinical tumour stage. The 8th edition of the AJCC manual addresses these issues, providing more accurate discrimination between groups and better risk stratification in patients with HPV-positive OPC. Piotr Machczyński et al,[39] pointed to the pros and cons and concluded that it represents a major improvement in group discrimination and risk stratification in patients with HPV-positive OPC. Some of the major changes for head and neck oncology in this 8th edition of AJCC included [40]:

- Oropharyngeal cancers (OPC) were staged based on p16 status
- Division of pharynx chapter into separate chapters for oropharynx and hypopharynx
- Changes were made to the nodal (N) staging with emphasis on extra nodal extension (ENE) increasing (N) staging in most subsites except in mucosal melanoma, nasopharyngeal carcinoma, p16+ oropharyngeal cancer

The T staging for both is very similar, but one main difference is that staging is present only in p16-negative disease. In addition, T4b has been removed from the p16 positive T staging as survival curves were indistinguishable for T4a and T4b in HPV related OPC (AJCC). There are differences in the nodal staging for p16 positive vs negative patients [41]. A unique staging system is in place for both clinical and pathological nodal staging for p16-postive OPC (AJCC) [41]. In contrast, nodal staging for p16-negative disease is similar to the previous head and neck cancer nodal staging with the addition of incorporating ENE, which is associated with a higher nodal staging (AJCC). Notably, ENE has been demonstrated to be an adverse pathological variable associated with increased rates of recurrence [42].

1.8 What is the best clinical method for HPV detection?

A variety of HPV detection methods are available (**see Figure 1**), however not all testing methods are equal, and this may explain the variations in different studies [43]. A widely used ‘surrogate’ biomarker of HPV activity, immunohistochemistry (IHC) staining of the p16INK4a protein (also referred to as simply, p16) is by far the most regularly implemented test for HPV diagnosis. p16 is a cyclin-dependent kinase inhibitor and is induced as a consequence of inhibition of Rb activity by the

HPV E7 oncoprotein [44]. p16 is consistently overexpressed in both cervical and oropharyngeal squamous cell carcinomas and correlates with better survival in patients with HNSCC [45] as noted previously. p16 IHC has a sensitivity of 94% (CI: 91-97%) and specificity of 83% (CI: 78-88%) [46].

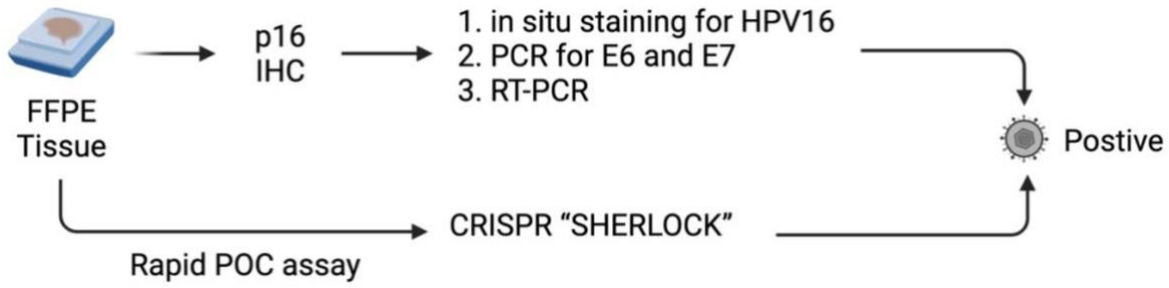

Figure 1. HPV detection methods. p16 immunohistochemistry testing for HPV DNA is generally the first point of call. If further confirmatory testing is required, methods such as in situ hybridisation for specific HPV stain HPV16, PCR for oncogenes E6 and E7 testing, and RT-qPCR for HPV mRNA can be undertaken. New methods using CRISPR technology has also been recently developed to detect HPV mRNA using a rapid point of care assay. Graphic prepared in BioRender.

A meta-analysis explored the differences between various HPV positive and negative phenotypes versus p16 positive and negative phenotypes to overall patient survival, identifying potential new biological subtypes in the HPV-related OPC aetiology. Patients that were found to be both p16 positive and HPV-positive had significantly improved five-year overall survival compared to patients who were both p16 negative and HPV-negative [47]. This data suggests that in some HPV positive, p16 positive cancers, HPV may be an innocent bystander, with p16 overexpression independently suggesting a biological cascade leading to cancer on its own accord, which is yet to be biologically characterised [48].

While the use of IHC p16 testing is simple and routinely utilised in clinical settings, a major caveat of using p16 as a biomarker is that a subset of tumours can be HPV positive but virally negative, where the loss of Rb activity has been shown not to be specific to the binding of HPV oncogene, E7 [49, 50].

Another methodology used in clinical settings is in situ hybridisation (ISH), which allows for the reliable detection of HPV in topographical relationship to the pathological lesion[51]. The detection procedure for ISH occurs within the nuclei of cancer cells. Through the process of hybridisation, probes bind to the DNA, and this is evaluated microscopically with the appearance of a precipitate indicative of a positive result [51]. In addition, the physical state of the virus can be evaluated by the presence of punctuate signals for integrated virus and diffuse signals for episomal virus. In this way, ISH may overcome some of the limitations of PCR by detecting only clinically relevant infections. While the specificity of this method is high (100%), the sensitivity is not ideal (83%) [52] due to multiple factors including high intra-observer variability, faint signalling, and non-specific staining.

Hospitals now advocate screening by p16 IHC followed by detection of HPV DNA, either by consensus PCR or In Situ Hybridisation [52, 53]. PCR primers used for HPV DNA detection have traditionally targeted the L1 region (among these, commonly commercially available primer sets include PGMY09/11, GP5+/GP6+ and SPF10 LiPA), however this region may be deleted during viral integration potentially reducing the sensitivity of this method of detection [54].

The clinical relevance of detecting HPV DNA is particularly important for head and neck tumours. The presence of latent virus leads to false positive results due to the ability of PCR to detect just a few copies of HPV DNA per cell [54]. Attempts have been made to resolve this issue through the use of real-time PCR, which provides a quantitative analysis of viral load and confirmation of a

transcriptionally active virus. In this way, RT-PCR amplification of viral E6/E7 mRNA is now considered the 'gold standard' for the detection of HPV infection within tumour specimens [55]. The method is reliable to both fresh frozen specimens, and FFPE samples [56].

Another methodology that has recently gained traction is the utilisation of CRISPR as a diagnostic tool for infectious diseases and cancer-associated mutations. In particular, the use of CRISPR associated protein (Cas) (CRISPR-Cas) which has been modified to enable the precise editing of virtually any DNA or RNA molecule. Kellner et al recently designed a CRISPR-based diagnostic tool that combined nucleic acid pre-amplification with CRISPR-Cas enzymology for specific recognition of disease DNA or RNA sequences [57]. Termed the "specific high-sensitivity enzymatic reporter unlocking" ("SHERLOCK"), this method allows for the multiplex, portable and ultra-sensitive identification of RNA or DNA from clinically applicable samples [57]. Impressively, a more recent DNA endonuclease-targeted CRISPR trans reporter (DECTR), a rapid (~30 mins), low cost and accurate CRISPR-Cas12 assay for the detection of viral infections [57] and may become the diagnostic tool of the future.

Despite these advancements, the technology requires precise control over conditions and reagents, which may pose logistical challenges in a typical clinical setting. Moreover, the need for specific training on CRISPR technologies and potential regulatory hurdles regarding the use of genetically engineered components can limit widespread adoption. While these assays offer significant sensitivity and specificity, they are still relatively new and must be validated through extensive clinical trials to confirm their efficacy and safety across diverse patient populations. Currently, the routine use of CRISPR assays in pathology departments remains limited, as more traditional methods are well-established and generally less resource-intensive.

1.9 Clinical perspective and de-intensification therapy for HPV-positive OPC

Clinically, HPV-positive OPCs have been shown to have a distinct prognosis, compared to HPV-negative tumours, particularly in regard to survival rates and responses to treatment [58]. Patients with HPV-positive tumours have better survival chances and improved prognosis, suggesting an underlying contribution by the presence of HPV.

Contemporary management of OPC involves patients being assessed in a tertiary referral hospital with a dedicated head and neck cancer team where patients are presented in a multidisciplinary setting. It should be noted that while this is the case for most patients, the need for tertiary referral is not an obligation for patients globally, where access to multidisciplinary teams can

vastly differ. The current edition of the AJCC stages patients depending on their p16 status in line with reported survival outcomes. Treatment recommendations are influenced by individual cases taking into consideration of disease factors, patient factors and institutional guidelines [58] These recommendations are based on the three main arms of cancer management: surgery, radiation oncology and medical oncology (which includes immunotherapy).

Traditionally single modality surgery was the mainstay of treatment (with or without adjuvant chemoradiotherapy) [59]. These operations were extensive and often left the patient with significant morbidity. Notably, the reliance on chemoradiotherapy to treat patients with OPC occurred before understanding the role of HPV and was associated with improvements in chemotherapy agents and improved delivery of radiation therapy. The role of HPV in the aetiology of OPC occurred in line with patients with HPV-driven OPC treated with chemoradiotherapy [60].

With improvements in chemotherapy agents with less toxicity and improvements in the delivery of radiotherapy (IMRT) and given the sensitivity of p16 positive OPC to chemoradiotherapy, the emphasis of treatment shifted towards a non-surgical modality. Surgery was reserved for persistent or recurrent disease. Generally, chemoradiotherapy is given concurrently with reported studies using different protocols (such as neoadjuvant chemotherapy followed by chemoradiotherapy). Although it should be noted that current research has not demonstrated a benefit in other treatment regimes.

With improvements in surgical techniques to reduce morbidity such as trans-oral robotic surgery (TORS), this approach has gained popularity. It was appreciated that several patients presenting with small volume primary disease (T1 and T2 disease) could be resected without significant morbidity and as such, surgery is an accepted alternative form of treatment for those with low volume disease at the primary site and limited regional nodal disease. This involves resection of the primary oropharyngeal tumour with a neck dissection. Some patients can be effectively treated with surgery alone.

In these patients, only half will require adjuvant radiotherapy based on adverse pathological features and is therefore seen as a form of de-intensification of treatment. However, in patients in which radiotherapy is required, the total treatment time is increased with surgery, surgery healing time and then adjuvant radiotherapy.

The dose of radiotherapy in this setting is generally lower and is associated with less toxicity and morbidity with the radiotherapy treating potential microscopic residual disease and gross disease

[61]. While TORS is considered by some as a form of treatment de-intensification due to the lack of morbidity compared with chemoradiotherapy, a recent phase 2 multicentre ORATOR trial has shown data suggesting swallowing outcomes were better for patients who were treated in the radiotherapy arm compared to TORS at the one year follow up [61].

De-intensification of chemotherapy has been considered in recent years for OPC. Two large phase 3 randomised trials (NRG Oncolocy RTOG 1016, DE-ESCALaTE) examined chemotherapy de-intensification by replacing cisplatin with cetuximab in HPV related/ p16 positive OPC patients. The outcomes of these two studies show no difference in adverse events between the two chemotherapy agents, hence there has been no proven data at this time to support chemotherapy de- intensification for OPC patients [60]. It was recommended that cisplatin and radiotherapy should be used as the standard of care for OPC HPV-positive low-risk patients.

One active clinical trial of note is the PATHOS study with 1100 participants; a phase II/III randomised control trial that examined reduced intensity adjuvant therapy following TORS [62]. In this study, patients were randomised into cohorts of no radiotherapy, adjuvant radiotherapy 50 Gy, adjuvant radiotherapy 60 Gy and adjuvant chemoradiotherapy. This trial is due to finish in 2026 and should provide a clearer outcome for de-intensification strategies. Previous trials substituting cisplatin with cetuximab have failed to show non-inferiority in terms of outcome and HPV status has no confirmed prognostic role for non-OSCC HNSCC [63].

Many centres may carry out primary tumour resection and neck dissection as one combined procedure. The ligation of the ipsilateral lingual-facial artery is also performed to minimise bleeding from the wound bed after resection of the primary tumour. Some centres would stage surgical intervention into two-stage surgery; firstly, a neck dissection followed by a second surgical procedure after seven days, for the resection of the primary tumour. This methodology was primarily to avoid communication of the pharynx with the neck.

While this is the standard approach, the disadvantage of having two procedures is that the patient requires two general anaesthetics with the associated risks. However, the benefit of having this staged procedure is that the patient would have a formal pathological staging of their neck disease first. For some patients, this may mean upstaging of their OPC and as such, primary surgery is no longer deemed appropriate, and they would go on to have staging appropriate treatment. Arguably, this is seen clinically as a treatment benefit, as these patients would have been otherwise undertreated.

The second advantage of two stage surgery is the perceived benefit of wound healing and granulation of the deep neck wound in the seven days before TORS which would minimise the risk of a continuous defect from the primary tumour wound bed out into the neck and causing an oro-cutaneous fistula (see supp table 1 for details on current HPV-related head and neck cancer clinical trials for de-escalation therapy and HPV detection).

For patients with advance HPV16 OPCs, several current trials are currently underway. The application of the immunotherapy drug Bintrafusp alfa (also called M7824), has demonstrated some benefit to patients with a larger Phase II trial for M7824 being considered. In the trial (NRG HN500), with accrual of 700 patients, this study will examine the benefits of concurrent reduced-dose radiation therapy with cisplatin or concurrent reduced-dose radiation therapy with nivolumab (immune checkpoint inhibitor) to the current standard of care (standard-dose RT with cisplatin).

1.10 Conclusions

The discovery and confirmation that HPV16 is a causative agent for certain subtypes of HNSCC has promoted a re-evaluation of our current treatments. The oropharynx (tonsil and base of tongue) are the main sites of infection and of concern are the increasing incidence in the younger population with little to zero tobacco exposure. The prognosis of HPV-mediated OPC is significantly better than that of the tobacco-induced malignancies.

Depending on the physician and hospital, treatments for OPC may vary slightly, and the presence of HPV16 may or may not influence treatment. More recently, the American Society of Clinical Oncology has recommended that radiotherapy followed by cisplatin treatment is the preferred approach for OPC p16/HPV positive cancers. For patients undergoing initial surgical resection (positive margins and/or extra nodal tumour extension), this will be followed by adjuvant chemoradiation with concurrent high-dose cisplatin. Patients with advanced nonoperative disease are recommended to undergo concurrent chemoradiation with high-dose cisplatin.

While de-escalating strategies may hold future promise, many of these studies have not provided the necessary data to support uptake for this modality of treatment Indeed one of the main criticisms is the design of the trials and the recruitment of appropriate patients. Precision medicine or the use of specific biomarkers may also guide future treatment decisions in HNSCC patients. For example, levels of HPV16 may guide the subsequent treatment paradigm after initial surgery or chemoradiation. Biomarkers to guide de-escalation of radiation therapy has had some success with a larger trial currently in progress. Most of these current and future treatments depend on the detection

of p16 as a surrogate marker for HPV positivity. Although it is widely accepted, there are also caveats to using this immunohistochemistry approach. The use of PCR technologies to detect viral particles does provide better specificity and sensitivity. Point of care technologies using CRISPR may be the future as these assays are highly specific with results know in less than an hour. The treatment of OPC p16/HPV positive cancers may change in the future depending on the results from all the trials and the development of new molecular assays. Instead of designing trials that assess the outcomes between surgical to non-surgical treatments, there is scope for trials investigating the use of neo adjuvants or adjuvant treatment. There would also be a shift to using HPV surveillance as a guiding tool in planning treatments. As the incidence of this disease is increasing, it is clear and evident that HPV16-related OPC are distinct entities from other HNSCC cancers, and their treatment will have to be re-evaluated in the coming years.

1.11 Supplementary material

Supplementary Table 1. Summary of HPV-related clinical trials for de-escalation therapy and HPV detection.

Clinical Trials Gov Identifier	Clinical Trial	Phase	Modality	Brief Commentary (results if available)	Status	Study Results	Conditions	Interventions
Deintensification studies								
NCT02002182	ADXS 11-001 Vaccination Prior to Robotic Surgery, HPV-Positive Oropharyngeal Cancer	Phase 2	Drug	The purpose of this study is to see if an experimental vaccine, ADXS11-001, is effective in stimulating the body's defence system against HPV-positive oropharyngeal squamous cell carcinoma before transoral (through the mouth) surgery. The experimental product ADXS11-001 uses a live strain of the Listeria monocytogenes (Lm) bacteria that has been genetically modified such that the risk of getting an infection is significantly reduced. Several research studies have already been conducted with ADXS11-001 in men and women with cancer. So far, approximately 722 doses of ADXS11-001 have been given to 290 patients with HPV associated cancers.	Active, not recruiting	Has results	Head and neck cancer Squamous cell carcinoma of the head and neck HPV positive oropharyngeal squamous cell carcinoma	Biological: ADXS11-001 (ADXS-HPV)
NCT04444869	Testing Less Intensive Radiation with Chemotherapy to Treat Low-risk Patients With HPV-positive Oropharyngeal Cancer (ENID)	Phase 2	Drug	This trial will explore giving standard dose chemotherapy and radiation therapy to sites of disease including all lymph nodes involved with HPV-positive oropharyngeal cancer but administer lower doses of radiation therapy to the lymph nodes that are not known to be involved with cancer. By doing so, it is hypothesized that there will be equally good long-term loco-regional and distant disease control but will reduced long term treatment side effects and improved quality of life in persons living well beyond their cancer treatment.	Recruiting	No results available	Cancer of the head and neck Oropharynx cancer HPV positive oropharyngeal squamous cell carcinoma Throat cancer	Drug: Cisplatin injection
NCT03210103	Primary Radiotherapy Versus Primary Surgery for HPV-Associated Oropharyngeal Cancer	NA	Radiation	The goal of this randomized treatment de-escalation study is to formally compare outcomes in HPV related oropharyngeal cancer tumours treated with a primary radiotherapy versus a primary surgical approach, to provide a high level of evidence to guide the selection of treatment options for a subsequent phase III trial. The study will compare overall survival rates relative to historical controls for de-intensified primary radiotherapy (60 GY +/- chemotherapy) versus transoral surgery (TOS) and neck dissection (+/- adjuvant 50Gy radiotherapy in patients with early T-stage HPV-positive squamous cell carcinoma of the oropharynx and to compare quality of life (QOL) profiles.	Recruiting	No Results Available	Oropharyngeal Cancer	Radiation; Procedure: Transoral Surgery (TOS) + Neck Dissection
NCT03777384	Evaluating the Safety of De-escalated Head and Neck Irradiation in HPV positive Oropharynx Cancer in Non-smokers/Minimal Smokers	NA	Radiation	This is a prospective, single-site, observational study in head and neck cancer participants. The purpose of this study is to provide a systematic platform to analyse, interpret, and track radiation dose de-escalation therapy and associated participant outcomes from treatments identified by these results.	Recruiting	No results available	Oropharyngeal cancer	NA

NCT03875716	Study of De-Intensified Postoperative Radiation Therapy for HPV Associated Oropharyngeal Squamous Cell Carcinoma	Phase 2	Radiation	This research study is studying lowering the standard dose of radiation and chemotherapy after surgery, to minimize the side effects and improve the quality of life.	Recruiting	No Results Available	Head and neck cancer Squamous cell carcinoma of the head and neck HPV positive oropharyngeal squamous cell carcinoma	Radiation: Radiation therapy
NCT03944915	De-Escalation Therapy for Human Papillomavirus Negative Disease	Phase 2	Drug/ Radiation	This study is looking to see if nivolumab, an immunotherapy drug, given with carboplatin and paclitaxel (2 chemotherapy agents) during induction therapy in advanced stage HPV negative patients can significantly shrink the subject's cancer.	Recruiting	No Results Available	Human Papilloma Virus Squamous Cell Carcinoma Squamous Cell Carcinoma of the Head and Neck HPV-Related Squamous Cell Carcinoma HNSCC	Drug: Carboplatin Drug: Paclitaxel Drug: Nivolumab Radiation: Radiation Drug: Hydroxyurea Pill Drug: 5-fluorouracil Drug: Filgrastim Injection Drug: Cisplatin
NCT03107182	Chemotherapy and Locoregional Therapy Trial (Surgery or Radiation) for Patients with Head and Neck Cancer	Phase 2	Drug/ Radiation	A phase II trial in human papillomavirus (HPV)-positive oropharyngeal squamous cell cancer (as determined by p16 immunohistochemistry with confirmatory ISH or PCR) to determine radiologic response to induction chemotherapy with nivolumab. Patients will undergo evaluation by a multidisciplinary team prior to risk assessment. The patients will be assigned to high or low risk groups based on tumour size, lymph node involvement, and smoking history. Patients will be assigned to treatment with induction chemotherapy with carboplatin, nab-paclitaxel, and nivolumab. Radiologic response to induction chemotherapy according to RECIST measurement of tumour shrinkage will then be used for therapeutic stratification of locoregional therapy, consisting of either transoral robotic surgery (TORS) or radiation with or without chemotherapy.	Active, not recruiting	No Results Available	HPV-Related Squamous Cell Carcinoma HNSCC	Drug: nab-paclitaxel Drug: Carboplatin Drug: Nivolumab Drug: Cisplatin Drug: Hydroxyurea Drug: 5-FU Drug: Dexamethasone Drug: Famotidine Drug: Diphenhydramine Drug: Paclitaxel Procedure: Transoral robotic surgery (TORS) Radiation: Adjuvant RT Radiation: Chemoradiotherapy
NCT01530997	De-intensification of Radiation & Chemotherapy in Low-Risk Human Papillomavirus-related Oropharyngeal Squamous Cell Cancer	Phase 2	Drug/ Radiation	The purpose of this research study is to learn about the effectiveness of using lower-intensity radiation and chemotherapy to treat human papillomavirus (HPV) associated low-risk oropharyngeal and/or unknown primary squamous cell carcinomas of the head and neck. The cure rate for this type of cancer is estimated to be high, > 90%. The standard treatment for this cancer is 7 weeks of radiation with 3 high doses of cisplatin. Sometimes surgery is performed afterwards. This standard regimen causes a lot of side effects and long-term complications. This study is evaluating whether a lower dose of radiation and chemotherapy may provide a similar cure rate as the longer, more intensive standard regimen. Patients in this study will receive 1 less week of radiation and a lower weekly dose of chemotherapy followed by a limited surgical evaluation.	Active, not recruiting	Has results	Carcinoma, Squamous Cell Head and Neck Neoplasms Oropharyngeal Neoplasms	Radiation: Intensity Modulated Radiotherapy (IMRT) Drug: Cisplatin Procedure: Limited surgical evaluation
NCT04502407	Trial of De-Intensified Post-operative Chemoradiation Following Robotic Surgery for HPV-positive Oropharyngeal Cancer	Phase 2	Drug/ Radiation	The study will assess whether a de-intensified version of standard chemoradiation treatment will be just as effective in treating HPV-associated oropharyngeal cancer while causing less side effects than standard dosing.	Active, not yet recruiting	Has results	Head and neck cancer Squamous cell carcinoma of the head and neck HPV positive oropharyngeal squamous cell carcinoma	Radiation: Cisplatin-based Radiation Therapy Drug: Cisplatin Chemotherapy

NCT03952585	De-intensified Radiation Therapy with Chemotherapy (Cisplatin) or Immunotherapy (Nivolumab) in Treating Patients with Early-Stage, HPV-Positive, Non-Smoking Associated Oropharyngeal Cancer	Phase 2/ Phase 3	Drug/ Radiation	This phase II/III trial studies how well a reduced dose of radiation therapy works with nivolumab compared to cisplatin in treating patients with human papillomavirus (HPV)-positive oropharyngeal cancer that is early in its growth and may not have spread to other parts of the body (early-stage) and is not associated with smoking. Radiation therapy uses high-energy x-rays to kill tumour cells and shrink tumours. Chemotherapy drugs, such as cisplatin, work in different ways to stop the growth of tumour cells, either by killing the cells, by stopping them from dividing, or by stopping them from spreading. Immunotherapy with monoclonal antibodies, such as nivolumab, may help the body's immune system attack the cancer, and may interfere with the ability of tumour cells to grow and spread. This trial is being done to see if a reduced dose of radiation therapy and nivolumab works as well as standard dose radiation therapy and cisplatin in treating patients with oropharyngeal cancer.	Recruiting	No Results Available	Basaloid Squamous Cell Carcinoma Clinical Stage I HPV-Mediated (p16-Positive) Oropharyngeal Carcinoma AJCC v8Clinical Stage II HPV-Mediated (p16-Positive) Oropharyngeal Carcinoma AJCC v8Oropharyngeal Squamous Cell Carcinoma Papillary Squamous Cell Carcinoma Pathologic Stage I HPV-Mediated (p16-Positive) Oropharyngeal Carcinoma AJCC v8Pathologic Stage II HPV-Mediated (p16-Positive) Oropharyngeal Carcinoma AJCC v8Squamous Cell Carcinoma	Drug: Cisplatin Radiation: Image Guided Radiation Therapy Radiation: Intensity-Modulated Radiation Therapy Biological: Nivolumab Other: Quality-of-Life Assessment Other: Questionnaire Administration
NCT03323463	Major De-escalation to 30 Gy for Select Human Papillomavirus Associated Oropharyngeal Carcinoma	Phase 2	Drug/ Radiation	The purpose of this study is to demonstrate that participants with HPV positive and hypoxia negative T1-2, N1-2c (AJCC, 7th ed.) oropharyngeal squamous cell carcinoma receiving a major de-escalated radiation therapy with 2 cycles of standard chemotherapy is not inferior to comparable subjects treated with the current standard chemoradiation.	Recruiting	No Results Available	HPV-Associated Oropharyngeal Squamous Cell Carcinoma Squamous Cell Carcinoma of the Neck	Diagnostic Test: F-FMISO PET/CT scan Radiation: 30 Gy over 3 weeks Drug: Cisplatin Drug: Carboplatin Drug: 5Fluorouracil Radiation: Proton Therapy
NCT01663259	Reduced-intensity Therapy for Oropharyngeal Cancer in Non-smoking HPV-16 Positive Patients	NA	Drug/ Radiation	The primary objectives include the following: to confirm that reducing treatment intensity in patients with HPV-related oropharyngeal cancer and < 10 pack-year smoking history by replacing concurrent chemotherapy with concurrent cetuximab, does not significantly increase the proportion of patients whose tumours recur, compared to our previous experience in similar patients receiving chemo-RT and to compare the toxicity in patients receiving cetuximab-RT to similar patients treated with 7 weeks of chemotherapy concurrent with RT ("standard therapy") in UMCC 2-21.	Completed	Has results	Squamous Cell Carcinoma of the Oropharynx HPV	Drug: Cetuximab Radiation: Radiotherapy

NCT03396718	De-escalation of Adjuvant Radio (Chemo) Therapy for HPV-positive Head-neck Squamous Cell Carcinomas	NA	Radio (chemo) therapy	In patients with squamous cell carcinoma of the oral cavity, the oropharynx and larynx with local advanced tumours (pathologic stage T3 = pT3) and or lymph node involvement (pN+) postoperative radio - or radio-chemotherapy is the standard of care. Postoperative radio-chemotherapy is indicated in patients with multiple lymph node metastasis, lymph node metastasis with extracapsular spread and / or residual tumor (R1-Status) after resection. Oropharyngeal cancer caused by HPV (human papillomavirus 16 or 18) is a distinct subgroup with a known sensitivity to radiotherapy (RTx) or radio-chemotherapy (RCTx). Additionally, a superior outcome after R(C)Tx over HPV negative patients was shown for patients treated with primary or adjuvant RCTx. To date it is unknown if the total dose of the radiotherapy can be safely reduced with the aim to decrease the therapy associated late effects. Patients with a HPV associated carcinoma that take part in the study will be treated with a reduced radiotherapy dose, chemotherapy will be prescribed based on clinical factors (number of affected lymph node, presence of extracapsular spread or residual tumour). Radiation dose will be reduced in two steps.	Recruiting	No Results Available	Head-and-neck squamous cell carcinoma	Radiation: De-escalation radio-chemotherapy - Level 1 Radiation: De-escalation radio-chemotherapy - Level 2 Radiation: Standard radio-chemotherapy
NCT04638465	De-Escalation Protocol of HPV Mediated Oropharyngeal Squamous Cell Carcinoma	NA	Robotic/ Radiation/ Drug	The purpose of this study is to evaluate the effects, good and/or bad of treating participants with HPV-mediated oropharyngeal cancer, with less treatment, using the new staging system. The investigators believe this treatment will provide the same effectiveness as the usual treatment but decrease the side effects. The radiation doses, chemotherapy doses, and the type of surgical approaches that will be used in this treatment protocol have all been previously investigated. Previous research suggests that this can be done safely, but there has not been a study done basing treatment on the new staging system.	Recruiting	No Results Available	HPV-Mediated (P16-Positive) Oropharyngeal Carcinoma by AJCC V8 Clinical Stage Oropharynx Cancer	Procedure: Transoral robotic surgery Drug: Cisplatin - Dose Level 1 Drug: Cisplatin - Dose Level 2 Radiation: Dose Level 1 Radiation: Dose Level 2
NCT02072148	The Sinai Robotic Surgery Trial in HPV Positive Oropharyngeal Squamous Cell Carcinoma (SCCA) (SIRS TRIAL)	NA	Robotic/ Radiation	There are currently few trials examining the role of de-escalation using surgery alone in intermediate and early T-stage HPV related disease. New surgical techniques have broadened the range of patients capable of achieving a complete resection and the functional outcomes in such patients are outstanding. Furthermore, the sensitivity of HPVOPC to chemotherapy and radiotherapy raise the possibility that delayed or salvage treatment in early-stage patients would be highly effective, would result in similar survival outcomes and radiotherapy could be applied to a much smaller population then current standards call for. Looked at from a different perspective, the need for post-operative radiotherapy in this younger, HPV+ and more functional population has not been validated in clinical trials to date.	Recruiting	No Results Available	Human Papilloma Virus Squamous Cell Carcinoma Squamous Cell Carcinoma of the Head and Neck HPV-Related Squamous Cell Carcinoma HNSCC	Procedure: PET/CT Radiation: Radiotherapy Radiation: Concurrent Chemoradiation
Completed HPV Screening Trials								
NCT01984359	HPV Serum DNA Levels Predicting Outcome p16+ Squamous Cell head and Neck Cancer	NA	-	The aim of this study is to assess in an exploratory manner, the prognostic utility for locoregional control, progression-free and distant metastasis-free survival of a pre-therapy and post-therapy blood DNA test of HPV E6 and E7 DNA for subtypes 16 and 18 in p16+ and/or HPV+ oropharyngeal cancer patients. This will entail analysis of both initial pre-therapy HPV level as a continuous variable and initial post-therapy HPV level as a dichotomous variable.	Completed	No results available	Subjects With p16 Positive/HPV Positive Squamous Cell Carcinomas of the Oropharynx	Biological: Blood sample collection Procedure: Tumour biopsies Other: CT scan Drug: Atezolizumab Drug: UCPVax

NCT02147418	Exosome Testing as a Screening Modality for Human Papillomavirus-Positive Oropharyngeal Squamous Cell Carcinoma	NA	-	Cancer of the oropharynx (middle, side, and back walls of the throat; back of the tongue; soft palate, and tonsils), or oropharyngeal squamous cell carcinoma (OPSCC), has been on the rise in the United States. Human papillomavirus (HPV) has been recognized in many of these cancers, and testing for HPV has contributed to the higher reported rates of OPSCC. In this study, the goal is to develop a new test that can detect certain HPV proteins in the blood or saliva to help improve detection of OPSCC.	Completed	No Results Available	OPC	The presence of unique proteins obtained from primary cell cultures derived from HPV-OPSCC confirmed patients will be compared to normal tonsillar epithelial cells and established cell lines. The distribution of these protein signatures will be compared in HPV-OPSCC and normal epithelial cells.
NCT01984359	HPV Serum DNA Levels Predicting Outcome in p16+ Squamous Cell Head and Neck Cancer	NA	-	To assess in an exploratory manner, the prognostic utility for locoregional control, progression-free and distant metastasis-free survival of a pre-therapy and post-therapy blood DNA test of HPV E6 and E7 DNA for subtypes 16 and 18 in p16+ and/or HPV+ oropharyngeal cancer patients. This will entail analysis of both initial pre-therapy HPV level as a continuous variable and initial post-therapy HPV level as a dichotomous variable.	Completed	No Results Available	Subjects With p16 Positive/HPV Positive Squamous Cell Carcinomas of the Oropharynx	Other: Obtaining Human tissue
NCT03226613	New Modalities for Detection of Oropharyngeal Cancer	NA	-	Objective/Hypothesis: To investigate two promising screening modalities for the detection of HPV-OPC, transcervical ultrasound and HPV16 E6 antibodies. The investigators hypothesize that both ultrasound and HPV16 E6 antibodies will be highly sensitive for the detection of symptomatic HPV-OPC. Specific Aims: (1) Determine the sensitivity of ultrasound to characterize OPC tumours compared to current standard imaging modalities among patients with suspected or confirmed OPC. (2) To determine the sensitivity and specificity of HPV16 E6 antibodies for HPV-OPC. (3) Determine the sensitivity of ultrasound to detect HPV-OPC compared to current standard imaging modalities among patients that present with a neck mass and unknown primary tumour.	Completed	No Results Available	Human Papillomavirus Positive OPSCC	Procedure: Transcervical Oropharyngeal Ultrasound Procedure: Oral Rinse Collection Procedure: Blood Draw

Chapter 2

The application of salivary non-coding microRNAs for the diagnosis of oral cancers

2.1 Copyright Information

This chapter has been published as an article in the journal of ***Head and Neck*:**
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The text presented here is the accepted version of the manuscript. Numbering of sections, style of referencing, number of tables and figures are altered to align with the formatting of the thesis.

2.2 Author Contribution

Authors: Fiona T Deutsch, Samantha J Khoury, John B Sunwoo, Michael S Elliot & Nham T Tran.

Fiona Deutsch (graduate student) is the first author of this Review article. Her contribution to this manuscript is demonstrated by the following roles/ tasks: researched the field, undertook a literature review, prepared the tables and figures, and was actively involved in the initial drafting, editing and review process.

Author permission to include this paper

I provide permission for Fiona Deutsch to include "Application of salivary noncoding microRNAs for the diagnosis of oral cancers" in her PhD thesis.

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2.3 Abstract

Oral cancer is on the rise globally and survival rates, despite improvements in clinical care, have not improved. Early detection followed by immediate intervention is key to improving patient outcomes. The discovery of microRNAs as biomarkers has attracted clinical interest but several challenges remain. These microRNAs can be found in bodily fluids including saliva which has been investigated as potential source of biomarker discovery. We undertook a systematic review of PubMed to identify studies that have characterised the salivary microRNAs present in patients with oral squamous cell carcinoma. Thirteen papers were considered for this review in accordance with their relevance to microRNA biomarkers, oral cancer, and saliva-based diagnostics. Our review provides an outline of the current advances for the application of salivary microRNAs in oral cancer. We also provide a technical guide for the processing of salivary RNAs to ensure accurate clinical measurement and validation.

2.4 Introduction

Oral cancer, representing a group of malignancies including the lip, anterior two-thirds of the tongue, gums, buccal mucosa, floor of the mouth, hard palate and minor salivary glands, is a late presenting disease linked to a high mortality rate over five years. It is distinct from pharyngeal cancers, which inolve the nasopharynx, oropharynx, and hypopharynx – areas where tumour behavious significantly difference, especially in terms of prognosis and treatment outcomes. GLOBOCAN estimated that oral cancers can be attributed to 529,5000 new cases and 292,300 deaths worldwide. This accounts for 3.8% of all cancer cases and 3.6% of cancer deaths [64].

It is well established that the treatment of early-stage oral cancers achieves higher survival rates with less morbidity. The five-year survival rate for oral cancer patients whose disease is detected early is 70%. This is compared to 37% five- year survival rates in cases of late diagnosis [65, 66]. These statistics are unfavourable but also define the solution; find an effective clinical biomarker for the early detection of oral cancer and this will save lives.

The treatment of advanced oral cancers is often associated with high morbidity as it affects organs critical in basic functions such as speech and swallowing. It is also commonplace that patients requiring surgery undergo major facial reconstruction, which greatly impacts appearance and quality of life. The standard diagnosis is often made by a tissue biopsy, which can cost in the range of USD $1,000-$3,000. Individuals from lower socioeconomic backgrounds and countries with inaccessible health-care systems may not have this choice. Furthermore, it is estimated that oral cancer-advanced patients incur three times more in treatment and care costs, than early-stage patients [67, 68].

Saliva is a water-based bodily fluid containing trace amounts of inorganic elements and heterogenous populations of biological particles. Saliva is primarily secreted by three major salivary glands (parotid, submandibular and sublingual), with an extensive blood filtering process occurring within highly specialised glandular cells. Post-filtering, an exchange occurs between the circulatory system and saliva, which explains how molecules that are present in plasma are also present in saliva. It is for this reason saliva has been termed the “mirror to the body,” as it reflects local and systemic conditions [69].

Saliva is needed in maintaining oral health, homeostasis, beginning the process of digestion

and providing lubrication and protection for the upper gastrointestinal tract from abrasion and wear [70]. This medium contains important enzymes protecting the oral cavity from disease by inhibiting the growth of bacteria and viruses. Over the last decade, advancement in transcriptomic techniques, quantitative PCR, array analysis and genomic sequencing has led to an interest in utilising salivary components as a diagnostic tool. This oral fluid has an abundance of biomarker material including hormones, interleukins, proteins and ribonucleic acids (RNA) subclasses. Globally, the common saliva- based biomarkers are hormones which are used for cortisol monitoring [71]. It can also be used for infectious disease testing, and substance abuse detection.

Though saliva has been widely suggested as a tool for screening, as it would be easily collected from a broader population [72], there are no commercially used or consistently reliable Food and Drug Administration (FDA, United States) approved saliva-based diagnostic methods. This review focuses on the research to-date for non-coding RNA (ncRNA) as saliva-based biomarkers in oral cancer. We review the current published approaches in saliva-based RNA methodology and put forward a set of suggested guidelines to standardise methods in collecting and analysing salivary biomarkers.

2.4.1 The non-coding RNAs family

Of the RNA family, non-coding RNAs (ncRNAs) are the most abundant type of RNA [73], equating to around 98% of transcriptional outputs in mammalian cells. These ncRNAs have been widely described as being stable in body fluids, ostensibly protected from RNA degradation, and are classified on the base of transcript size; small ncRNAs are less than 200 nucleotides and long ncRNAs are more than 200 nucleotides in length. Among all ncRNAs, small ncRNAs are the most exploited and widely described ncRNAs in saliva. Particularly, microRNAs (miRNAs), which are an RNA subclass of short, non-coding sequences (19-23-nucleotide long, single-stranded RNA molecule). These miRNAs act as regulators for a diverse range of physiological functions, including playing a role in oncogenesis and tumour progression (**Figure 1**). Their expression in circulation may be indicative of a cancer phenotype, and are touted as potential pathological tools for low-invasive cancer staging and prognosis [74]. MicroRNAs have been shown to be stable in blood circulation [75] but are also resistant to various environmental conditions [76]. Considering that oral cancers occur in the oral cavity, there is a likelihood that potential disease markers may drain into this fluid. To this end, recent studies have suggested the application of salivary miRNAs as biomarkers for cancer diagnosis [77, 78].

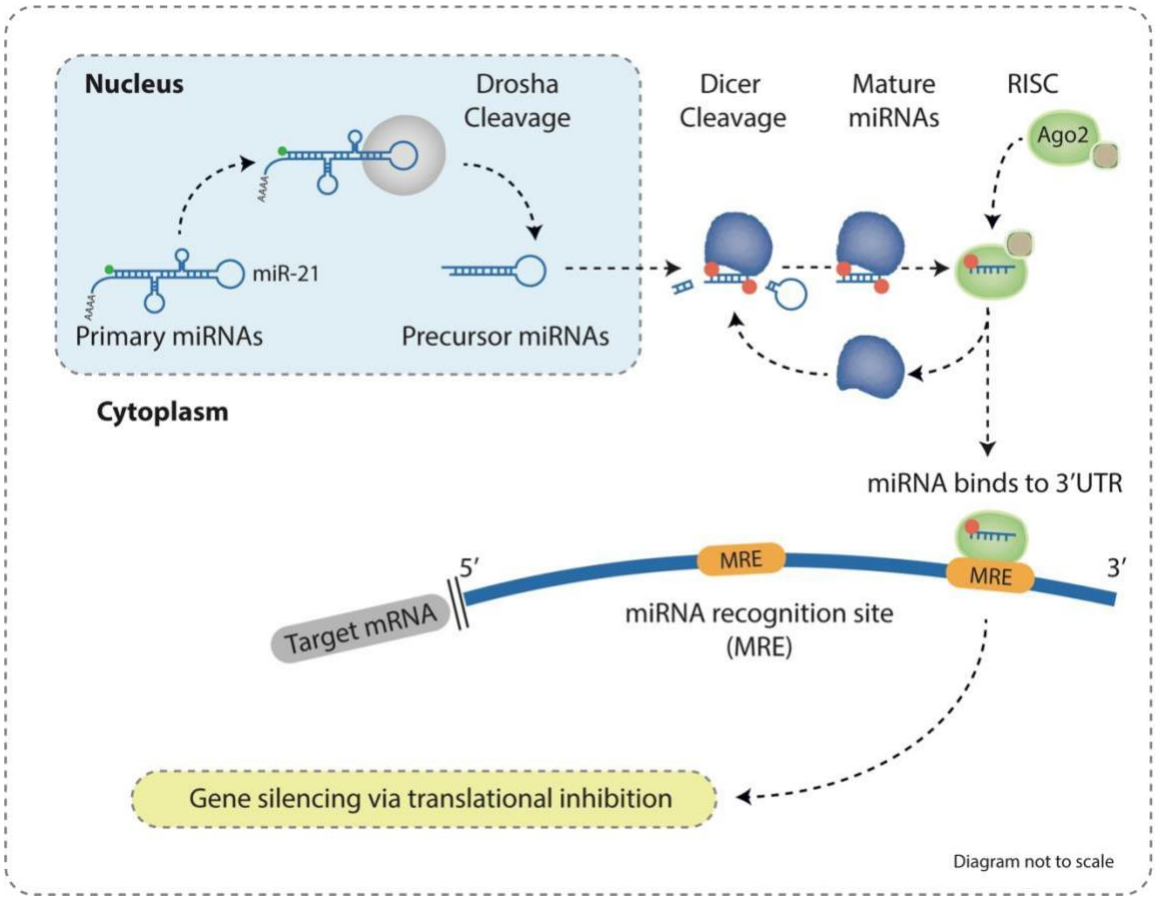


Figure 1. The Micro-RNA (miRNA) biogenesis pathway. In brief, miRNAs are transcribed by RNA polymerase II to produce the primary miRNA strand. These structures typically contain a hairpin which is then cleaved by Drosha in the nucleus to produce a precursor miRNA. This precursor is shuttled to the cytoplasm whereby it undergoes further processing. The enzyme Dicer cleaves the precursor miRNAs to produce the double-stranded RNA duplex (miRNAs). This miRNA is then loaded onto Ago2which is part of the RISC unit. This complex separates the two strands of the miRNAs leaving the sense or "guide" strand bound to Ago2. Using the guide strand, complementary binding occurs at the 3'UTR, which results in gene regulation via translation inhibition.

The major advantages of using saliva include the ease of collection, as it is non-invasive and requires no extra costs for trained personnel or complex procedures. There are however some caveats; saliva has low RNA abundance, and a small sample collection is common to patients with oral cancer. Of note, saliva contains highly fragmented mRNA and an abundance of bacterial content [79]. Despite these challenges, several groups have investigated the miRNA population within saliva.

2.4.2 Characterisation of salivary microRNAs

Weber and colleagues [80] examined the presence and distribution of miRNAs in 12 human body fluids, including saliva. Notably, saliva, breast milk and seminal fluid had the highest number of miRNA species. Interestingly, hsa-miR-509-5p, hsa-miR-515-3p and hsa-miR-335 were among the most abundant miRNAs found and shared among the different fluids, which suggests a common origin or function. In other studies, hsa-miR-223, hsa-miR-191, hsa-miR-16, hsa-miR-203, and hsa-miR-24 were found to be the five most abundantly miRNAs in saliva. Of note, RNA in these saliva samples were found to be stable for 48 hours at room temperature despite the enzymatic nature of saliva [81-84]. The salivary RNA transcriptome was measured in both whole saliva and cell-free supernatant (CFS) using RNA-Seq [79]. Whole saliva, the unaltered fluid obtained immediately after spitting contained more microbial sequences due to the higher bacterial content. In contrast, salivary supernatant obtained through centrifugation had more human transcripts. While there is a preference to use cell-free supernatant to reduce microbial RNA, a higher sequencing depth combined with an efficient PCR should also reduce the impact of these microbial RNAs. Other ncRNA families such as piwi-interacting RNAs (piRNAs), circular RNAs (circRNAs) and the transfer RNA (tRNA)- derived RNA fragments (tRFs) were shown to be present in the cell-free supernatant of saliva [85, 86].

2.4.2 Current status: Salivary microRNAs in oral cancer

The accumulated evidence clearly supports the notion of using miRNAs for diagnosis but to date, this has not been a clinical reality. For oral cancer, tissue-based profiling has been heavily invested by both researchers and companies (as reviewed by Troiano et al [87]). Implementing a miRNA tool into clinical practice has not been effectively disseminated,

though many expression signatures have been identified. A direct example are the numerous and public domain studies reporting associations between miRNA biomarkers and oral cancer [88-103]. These studies derived from profiling solid tumours, indicate miRNAs as highly stable in tissue. The discovery of blood-based or circulating miRNAs has expanded the search for other diagnostic mediums utilising miRNAs. Thus, the vital question is: can we harness the presence of miRNAs in saliva as markers for the diagnosis and prognosis of patients presenting with oral cancer?

Saliva-based miRNAs are ideal biomarkers as they can be readily collected without the need of specialised medical for equipment [104]. Due to the direct contact between saliva and the oral cancer lesion, it is possible that disease-related concentration changes of saliva components may provide better clues than systemic blood samples [105]. Saliva constituents, including proteins [105-109] and RNA [110] have been profiled in multi-stage cohorts and several miRNAs such as hsa-miR-187, hsa-miR483-5p and hsa-miR-9, are already proposed as diagnostic markers for oral cancer [110-112]. There are however limited studies of oral cancer biomarkers in saliva and their use for patient management [113]. A summary of the current methodologies and clinical studies is provided in **Table 1 and 2**, respectively.

The first investigation into salivary microRNAs found significantly lower levels of hsa-miR-125a and hsa-miR-200a in oral cancer when compared to healthy controls [82]. This study set the foundation to explore the potential of using salivary miRNAs as diagnostic markers in oral cancers. Lui et al [114] reported that hsa-miR-31 was upregulated by 10.2 fold in pre-surgery oral cancer patients compared with levels after resection. Interestingly, levels of hsa-miR-31 were more abundant in salivary supernatant than in plasma. Of note was that oral verrucous leukoplakia patients showed no difference in expression to the control group. In precancerous oral lesions, hsa-miR-10b, hsa-miR-145, hsa-miR-99b, hsa-miR-708, hsa-miR-181c, hsa-miR-30e, hsa-miR-660 and hsa-miR-197 were all upregulated compared to controls [115]. However, these profiles were measured using stimulated saliva through the use of mouthwash and it has been suggested that salivary stimulants can promote the release of non-associated microRNA species from the salivary glands [116].

Table 1. Summary of methodologies used in salivary miRNA biomarker literature.

Authors	Aim	Compartment	No. samples in cohort	Saliva collection method	RNA Isolation	RNA quantification	miRNA detection	MIQE	Validation method	Reference gene	miRNAs	Statistical analyses
Park et al 2009. (82)	miRNA expression in OSCC	Whole Saliva and Salivary Supernatant (Centrifuged at 2,600g x 10mins)	**n= 100**; 50 OSCC, 50 healthy	Unstimulated whole saliva - Volume not stated	*mir*Vana miRNA isolation kit (Ambion Inc.)	NA	qRT-preamp-PCR (Taq Man)	No	Preamp qRT-PCR (Taq Man)	U6 snRNA	hsa-miR-200a hsa-miR-125a	Mann Whitney U test, ROC analysis
Liu et al 2012. (114)	miR-31 expression in OSCC	Salivary supernatant (Centrifuged at 2,600g x 15mins)	**n= 74**; 45 OSCC, 10 OVL; 24 healthy	Whole saliva - Collected from floor of mouth after simple mouth rinse with water (5mL)	*mir*Vana PARIS miRNA isolation kit (Ambion Inc.)	NA	qRT-PCR (Taq Man)	No	Not stated	miR-16	hsa-mir-31	Mann-Whitney, Wilcoxon, linear regression, receiver operating characteristics (ROC analysis
Yang et al 2013. (115)	miRNA expression in precancerous oral lesions	Whole saliva	**n= 22**; 8 progressing LGD leukoplakias; 7 non-progressing LDG leukoplakias; 7 healthy volunteers	2mL whole saliva collected, immediately after mouthwash. Sample mixed with 5mL RNA protect saliva reagent (Qiagen)	RNeasy Micro Kit (Qiagen)	Nanodrop ND-1,000	TaqMan MicroRNA Assay (Applied Biosystems)	No	qRT-PCR (Taq Man)	RNU6	hsa-miR-10b, hsa-miR-145, hsa-miR-99b, hsa-miR-708, hsa-miR-181c, hsa-miR-30e, hsa-miR-660, hsa-miR-197	Random variance t-test, Benjamini-Hochberg false discovery rate (FDR) method, Mann-Whitney U test, student's t-tests (2-sided)
Salazar et al 2014. (117)	miRNA expression in OSCC	Whole saliva	**n= 122**; 61 HNSCC stages II-IV; 61 healthy controls	Whole saliva (unstimulated) - volume not stated	NucleoSpin miRNA Kit (Qiagen)	NA	miScript miRNA PCR arrays (Qiagen)	No	qRT-PCR (SYBR Green)	SNORD96A	hsa-miR-9, hsa-miR-191, hsa-miR-134	Double delta CT, normalisation (using SNORD96A), Mann Whitney U-Test, ROC analysis
Momen-Heravi et al 2014. (121)	miR expression in OSCC, OSCC-remission, oral lichen planus	Salivary supernatant (Centrifuged at 2,600 x 15mins)	**n= 34**; 9 OSCC; 8 OSCC-Remission; 8 OLP; 9 Health controls	Whole saliva (unstimulated) - maximum 8mL	Rneasy Kit (Qiagen)	Nanodrop ND-2000	Nanostring nCounter miRNA Assay (NanoString Technologies)	No	qRT-PCR (Taq Man)	miR-191	hsa-miR-27b	1-way ANOVA, 2-tailed Mann Whitney U test, ROC analysis
Zahren et al 2015. (118)	miRNA expression in OSCC, PMD	Salivary supernatant (Centrifuged at 2,500g x 10 mins)	**n=100**; 20 clinically healthy controls; 40 patients with oral PMDs; 20 with confirmed PMD that had not transformed to OSCC over at least a 3-year period (dysplastic lesions); 20 with PMD with non-dysplastic lesions	Whole saliva - volume not stated	microRNA isolation kit (QIAgen)	Nanodrop ND-1,000	qRT-PCR (SYBR-Green)	No	Not stated	SNORD68	hsa-mIR-21, hsa-miR-184, hsa-miR-145	Descriptive (mean/ SD/ SE/ range/ 95% CI), One-way ANOVA (compare mean of groups), Dunnett t-test (compare each group to control), Scheffe's multiple comparison method (compare means of each 2 groups), Significance, ROC analysis AUC
Duz et al 2016. (120)	miRNA expression in TSCC	Salivary supernatant (Centrifuged at 2,600 x 15mins)	**n= 50**; 25 TSCC taken prior to and once after surgical treatment; 25 healthy controls	Whole saliva, collected 1hour after participants were asked to stop eating and drinking	*mir*Vana PARIS miRNA isolation kit (Ambion Inc.)	Nanodrop ND-2000c	Agilent 8 x 60K human v19 miRNA microarrays	No	qRT-PCR (Taq Man)	RNU6b	hsa-miR-139-5p	2-sided students t-test, received operating curve, 95% confidence interval

Abbreviations: Oral squamous cell carcinoma, OSCC; Oral verrucous leukoplakia, OVL; Low grade dysplasia, LGD; Head and neck squamous cell carcinoma, HNSCC; Oral lichen planus, OLP; Potentially malignant disorder, PMD; Tongue squamous cell carcinoma, TSCC.

Table 2. Summary of the salivary miRNA biomarker results from the literature.

Authors	Total no. patients in cohort	No. patients with HNSCC	No. control specimens	Other cohorts included no. specimens	miRs of interest	Upregulated/ Downregulated (compared to healthy controls)	AUC	AUC characteristics scale: Fail - Excellent*	Combined AUC	Sensitivity (%)	Specificity (%)
Park et al 2009	100	50	50	0	miR200a	Downregulated	0.65	Poor	0.66	Not stated	Not stated
					miR-125a	Downregulated	0.62	Poor		Not stated	Not stated
Liu et al 2012	79	45	24	10 OVL	miR-31	Upregulated	0.82	Good	Not stated	80	68
Yang et al 2013	22	8	7	7 nonprogressing LDG leukoplakias	miR-10b	Upregulated	Not stated	Not stated	Not stated	Not stated	Not stated
					miR-145	Downregulated	Not stated	Not stated		Not stated	Not stated
					miR-99b	Downregulated	Not stated	Not stated		Not stated	Not stated
					miR-708	Upregulated	Not stated	Not stated		Not stated	Not stated
					miR-181c	Downregulated	Not stated	Not stated		Not stated	Not stated
					miR-30e	Upregulated	Not stated	Not stated		Not stated	Not stated
					miR-660	Upregulated	Not stated	Not stated		Not stated	Not stated
					miR-197	Downregulated	Not stated	Not stated		Not stated	Not stated
Salazar et al 2014	122	61	61	0	miR-9	Upregulated	0.85	Good	0.74	Not stated	Not stated
					miR-191	Upregulated	0.98	Excellent		Not stated	Not stated
					miR-134	Downregulated	0.74	Fair		Not stated	Not stated
Momen-Heravi et al 2014	34	9	9	8 OSCC remission, 8 OLP	miR-27b	Upregulated	0.9643	Excellent	Not stated	Not stated	Not stated
Zahren et al 2015	100	20	20	20 PMD with dysplasia, 20 PMD without dysplasia, 20 RAS	miR-21	Upregulated	0.73	Fair	Not stated	65	65
					miR-184	Upregulated	0.86	Good		80	75
					miR-145	Downregulated	0.68	Poor		60	70
Duz et al 2016	50	25	25	0	miR-139-5p	Downregulated	0.805	Good	Not stated	Not stated	Not stated

*In accordance with the suggestions from Hanley and McNeil, 1982.

Abbreviations: Oral squamous cell carcinoma, OSCC; Oral verrucous leukoplakia, OVL; Low grade dysplasia, LGD; Head and neck squamous cell carcinoma, HNSCC; Oral lichen planus, OLP; Potentially malignant disorder, PMD; Tongue squamous cell carcinoma, TSCC.

Several studies have investigated whole saliva, identifying hsa-miR-9, hsa-miR-191 and hsa-miR-134 [117]. In contrast, profiles using salivary supernatant from oral cancer patients identified hsa-miR-27b as having the highest sensitivity and specificity when discriminating oral cancer patients from the other groups. Notably, the expression of plasma and serum hsa-miR-27b was significantly reduced in oral cancer patients. This may support the idea that oral cancer cells directly secrete specific miRNAs into the oral cavity. Other studies using supernatant salivary indicated that hsa-miR-21, hsa-miR-184 were significantly higher in oral cancer patients and in oral potentially malignant disorders (OPMDs) than in healthy controls. In contrast, hsa-miR-145 was significantly lower in OSCC and OPMDs [118]. ROC analysis demonstrated sensitivity of 65%, 60% and 80% and specificity of 65%, 70% and 75%, for these three miRNAs respectively [119]. The latest study validated low expression of hsa-miR-139-5p in 50 saliva samples. ROC analysis with this miRNA discriminated between two groups of patients; asymptomatic and pre-treatment patients versus postoperative patients, with a value of 0.713 [120].

From all these studies, there is no general consensus regarding which miRNAs are suitable as biomarkers. Much of this variation is due to the different laboratory practices in collection, processing and detection of these miRNAs. These are the major obstacles for the application for any salivary-based miRNAs. Standardisation coupled with automation will go towards eliminating variation between laboratories and errors associated with manual handling of the specimen. The field of salivary miRNA biomarkers should adopt standardised guidelines for specimen collection, processing saliva and miRNA isolation techniques.

2.5 A framework for standardising miRNA isolation and detection from saliva

There are three forms of collection which are consistently used in the major studies.

(a) Typically, unstimulated cell-free supernatant (CFS) is the cleanest choice for RNA extraction. Collection is achieved by asking the patient to remain seated with no food or stimulating material, such as mouthwash, for at least 30 minutes prior. The patient is asked to clean their mouth with three to five millilitres of sterile water for 30 seconds, either swallowing it or spitting it out. After pooling saliva in their mouth, the patient or volunteer

can then expectorate into a specimen container where upon it is snap frozen. The saliva is centrifuged at 2,600g's for 15 minutes at 4^0Celsius to remove any cells and other debris, and the supernatant is obtained, ready for RNA analysis. Studies which have used this method include Park et al. [82], Liu et al. [114], Momen-Heravi et al. [121], Zahran et al. [118], and Duz et al [120].

(b) The second collection method uses unstimulated whole saliva (UWS), which is not desirable. Similar to unstimulated CFS, saliva is collected without the use of stimulating materials and is pooled in the mouth. The patient is then asked to expectorate into a specimen container for immediate snap freezing. Whole saliva is processed without centrifugation, however studies have noted the high presence of microbial RNA within whole saliva that has negative impacts on the sensitivity of human RNA detection [79]. Studies which used this method include Park et al [82] and Salazar et al [117].

(c) The last approach is to collect stimulated whole saliva. Chemical stimulation of saliva production may contaminate the oral cavity and alter the desired biological population of interest. Only one study [115] used this particular method.

Considering all of the above, our recommendations for collecting saliva for miRNA biomarker research are summarised in **Figure 2**.

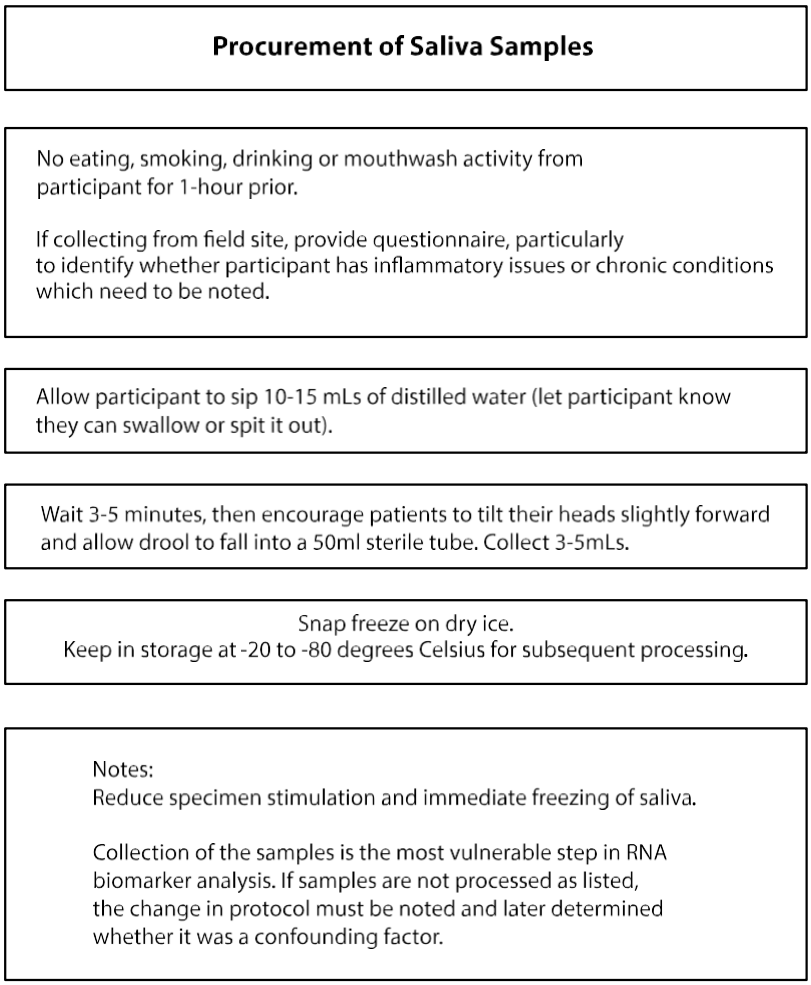


Figure 2. Procurement of saliva samples. Recommendations for collecting saliva for RNA-based biomarker research.

2.6 Extracting RNA from saliva

There are various approaches to isolating miRNAs ranging from liquid base guanidine isothiocyanate to silica columns [122]. All have merits, but a consensus must be reached, ensuring consistency between different cohort studies. **Table 1** refers to methodologies utilised by various research papers. Consistency of RNA extraction is important to eliminate study discrepancy and bias. It has also been shown that silica columns do not efficiently bind RNAs smaller than 200 nucleotides [123].

With this in mind, our laboratory has adopted a liquid-based guanidine isothiocyanate approach for the isolation of all RNA species from biofluids. We have optimised this protocol to deliver the highest yield of RNA from bodily fluids [124]. Recent studies have shown RNA profiles from non-invasive fluids such as saliva do in fact exhibit large variations in the RNA content and integrity [125]. Whole saliva has elevated but inconsistent levels of RNA content, similar to whole blood. Both mediums, have high numbers of cells and bacterial content, are at risk of false RNA quantification due to contamination with bacterial cells. Whilst RNA can be visualised by a distinct peak at 260nm, due to the low RNA content from saliva, the common profile exhibit is a flat reading over the visible spectrum, (**Supplementary Figure 1**). The suggested protocol is shown in **Figure 3**.

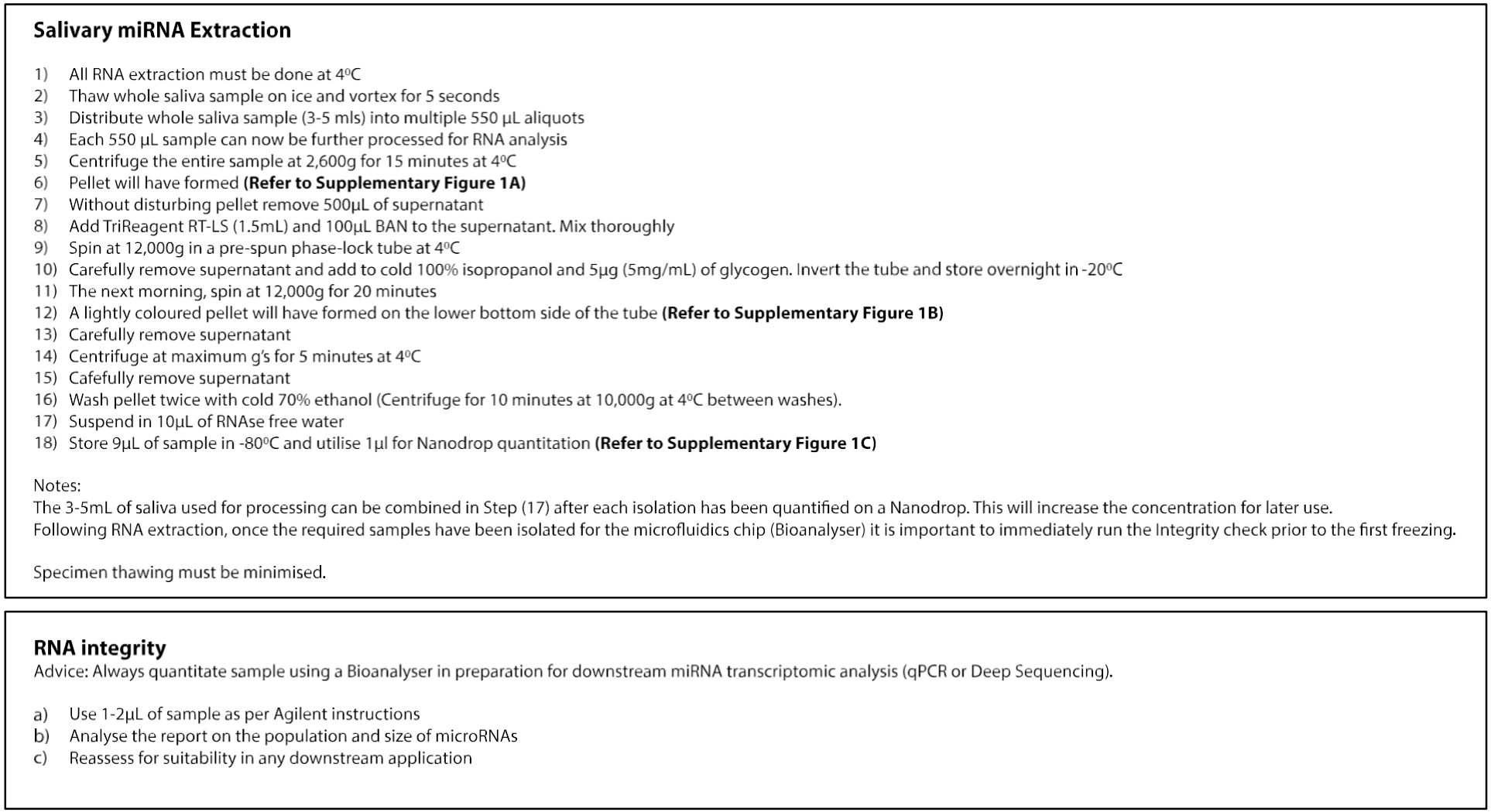


Figure 3. Proposed method to standardise salivary RNA extraction for miRNA analysis.

2.7 Lessons in saliva assay development

Several publications elucidated the role of miRNA transcripts deregulated in the general field of Head and Neck Squamous Cell Carcinomas [17, 82, 98, 101, 126, 127]. Following this, numerous articles reported the accuracy levels at which circulating miRNA biomarkers can precisely characterize cancer states in a diagnostic or prognostic capacity. Rationally, it can be expected that a suite of identified saliva miRNAs is of greater appeal than current techniques, as routine saliva samples are the only requirement for the diagnosis. However, there does not appear to be any consistency with recent studies unable to identify a set of miRNAs for oral cancer diagnosis. This is in part due to the various methods employed at the collection, processing and detection of these miRNAs.

Adding to these concerns is the difficulty associated with normalizing low miRNA expression in bodily fluids. Whilst global normalisation and reference genes dominate the reporting of qPCR experiments, difficulties are encountered when utilising samples with low RNA concentrations. Two approaches which are currently reported in the literature include the standardisation of qPCR data-handling pipelines [128] and the use of LinRegPCR [129]. Using an iterative algorithm, LinRegPCR determines baseline fluorescence, then creates a window of linearity (W-o-L) for each miRNA target. Following this it calculates the PCR efficiency per sample. The algorithm also calculates the Cq value and the starting concentration per sample. Utilising LinRegPCR to measure the constant amplification efficiency in all samples are vital. This allows the user to accurately measure any changes in miRNA expression without normalisation of the data to an external housekeeping gene or global normalisation. The consolidation of a universal miRNA extraction and detection pipeline will directly impact our ability to translate these miRNAs into the clinic. It is to-date the biggest challenge in the field.

2.8 Discussion and Conclusion

Biomarkers are important clinical tools for improving the survival rate of patients with head and neck squamous cell carcinoma. Traditionally, tissue, blood and urine were the preferred mediums for cancer detection. However, the rapid development of genomics and our increased understanding of molecular pathways has broadened the use of biomarkers to non-coding RNAs. As oral cancers originate in the oral cavity, it may be possible that saliva contains specific biomarkers reflective of this disease. Saliva has been widely suggested as a tool for screening, as it would be easily accessible

for a broader population [72]. Given the rising global incidence of oral cancers and the increased use of tobacco and alcohol, there is a requirement to develop better diagnostic tools.

Early detection is paramount for increasing survival, but most patients present with advanced TNM stage III or IV tumours. Conventional oral examination (COE) for many decades has been the most widely used and accepted screening method for oral cancers. There are some caveats with COE; the physical examination cannot identify all oral premalignant lesions and cannot predict which lesions will progress to cancer. As the test is conducted by trained individuals, the performance and diagnostic accuracy of COE may be dependent on clinical experience. The discovery and use of salivary miRNAs as a standardised test would add value for the early detection of this cancer. It may be that COE used in combination with a salivary test can increase the early detection rate of these cancers. Beyond the scope of early detection, these miRNAs could also be utilised as markers for disease monitoring, progression, and recurrence.

Disease monitoring may have scope through the use of saliva-based microRNA biomarkers. These miRs of interest are evaluated before and after treatment, and during patient follow-up. Salivary hsa-miR-31 was found to be significantly increased in patients with oral cancer at all clinical stages, including very early stages [114]. It was shown to be more abundant in saliva than in plasma, and after tumour surgical removal, its expression was reduced. However, the researchers found no difference between miRNA levels in premalignant lesions and healthy controls, indicating that further studies with larger cohorts are required to elucidate the status of hsa-miR-31 expression in precancerous stages. Despite this, salivary hsa-miR-31 remains a promising marker for early detection and postoperative follow-up of oral cancer and has potential for use in disease monitoring.

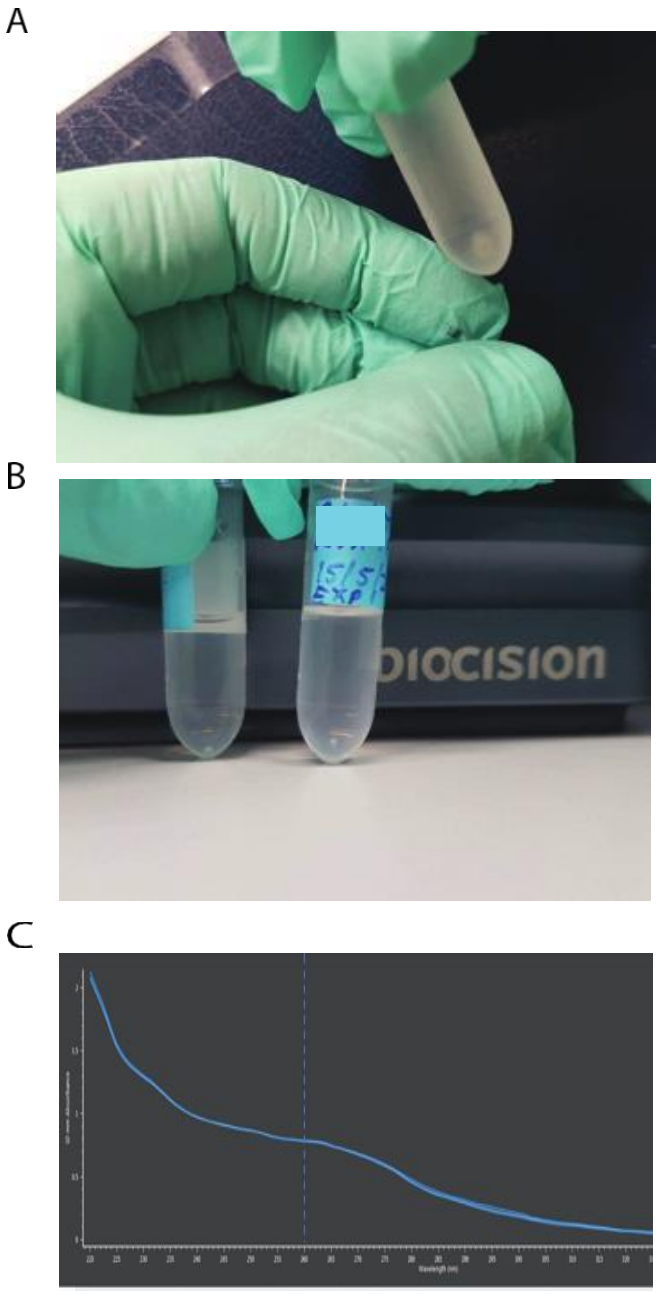
The set of salivary miRNAs identified in our review are different between dysplasia and cancer indicating that there are specific miRNA signatures which can denote the progression of the precancerous lesions. Yang et al demonstrated that salivary miRNAs were differentially expressed between low-grade dysplasia (LGD) and high-grade dysplasia (HGD) leukoplakia [115]. This would suggest that specific miRNA patterns can be used to follow disease progression from LGD to HGD which would result in early detection and clinical intervention.

Clinical markers which can predict disease recurrence are needed to effectively treat oral cancer patients. Approximately 30 to 40 percent of patients develop recurrent locoregional cancer, whereas between 20 to 30 percent will develop recurrent metastatic disease. Our review did not identify any salivary miRNAs which have been investigated as markers of recurrence. However,

studies using whole blood were able to define cut-off points to determine disease recurrence [130, 131]. In solid tumour studies, hsa-miR-194 and hsa-miR-99 displayed a significant correlation with local recurrence and progression-free survival [132]. As salivary miRNA research is just in its infancy, we believe there is scope to further investigate salivary miRNAs as markers of recurrence. This would require longitudinal studies to monitor patients over 3-5 years combined with RNA sequencing to find these miRNAs.

Our review offers a summary of the current state of salivary miRNAs and its potential applications to oral cancer. There are however limited studies in the area of salivary markers. We found that each study adopted a different methodology for processing and measuring salivary miRNAs. This fact alone makes it very difficult to compare studies and determine the accuracy of these reported miRNA biomarkers. This lack of standardisation in saliva processing will without doubt affect the reproducibility of results. It is clear that standardising saliva biomarker methodology is paramount for this field of research. Towards this end, we have presented a proposed guide for the collection and processing of miRNAs in saliva. The adaptation of a universal and common methodology would reduce the variations found in all current studies and bring us closer to discovering potential salivary miRNAs for use as clinical tools for oral cancer diagnosis.

2.9 Supplementary material



Supplementary Figure 1: A. Salivary pellet after 2,600 x g centrifugation for 15 minutes at 4°Celsius. Carefully remove the supernatant surrounding this pellet for processing. **B.** Salivary RNA pellet once centrifuged after overnight precipitation with 100% isopropanol and 5µg/ µL of glycogen. **C.** A typical spectrophotometric profile of total RNA extracted from human saliva.

Chapter 3

A Multiplex Quantitative PCR Liquid Biopsy to Detect Viable Human Papillomavirus 16 from Saliva

3.1 Copyright Information

This chapter is under review in the journal of ***BMC Cancer***.
The text presented here is the current version of the manuscript. Numbering of sections, style of referencing, number of tables and figures are altered to align with the formatting of the thesis.

Author Contribution

Authors: Fiona T Deutsch, Dayna Sais, Meredith Hill , Michael S Elliott, Nham T Tran.

Fiona Deutsch (graduate student) is the first author of this Research article. Her contribution to this manuscript is demonstrated by the following roles/ tasks: researched the field, undertook a literature review, undertook laboratory experimentation, prepared the tables and figures, and was actively involved in the initial drafting, editing and review process.

Author permission to include this paper

I provide permission for Fiona Deutsch to include "A Liquid Biopsy to Detect Transcriptionally Active Human Papillomavirus 16 From Patient Saliva" in her PhD thesis.

Signatures

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A/ Prof Michael S Elliot, MD, MPhil. — Production Note: Signature removed prior to publication

A/ Prof Nham T Tran, PhD — Production Note: Signature removed prior to publication

3.2 Abstract

There has been a significant increase in sexually-transmitted human papillomavirus (HPV) infections associated with head and neck cancers, particularly oropharyngeal cancers (OPC). HPV16 is a primary driver of cancer progression in OPC. We have developed and evaluated a multiplex approach that accurately differentiates between HPV positive and negative specimens in OPC patient tissue and saliva. The study successfully detected transcriptionally active E6 and E7 mRNA transcripts in HPV16 positive cell lines with a mean PCR efficiency of approximately 1.8, indicating optimal PCR amplification. Multiplexing assays for E6 and E7 in p16 positive OPC patient tissues showed an association between increasing levels of mRNA expression and increased RNA input. While there was some variability in PCR efficiency, the average remained above 1.7. Transcriptionally active E6 and E7 mRNA was only found in p16 positive patient saliva samples, confirming assay specificity. The E6 assay performed slightly better, with a mean Cq value of 31.5, compared to the E7 assay (mean Cq value 34.4). Through multiplexing, we have reduced sample input without loss of specificity. Furthermore, this assay measures RNA, which is an indication of a viable infection. Overall, this multiplex method provides a promising approach for accurately detecting HPV16 in OPC saliva and tissue. Its successful implementation could lead to improved clinical decision making and treatment planning for patients with HPV-related OPC.

3.3 Introduction

The underlying mechanisms responsible for head and neck cancers (HNC) are multifaceted and encompass both endogenous and exogenous risk factors. Tobacco and alcohol consumption are widely recognised as the primary risk factors for HNC [133]. The synergistic effect of tobacco and alcohol consumption on HNC has been extensively documented. However, human papillomavirus (HPV) infection has been implicated as a key determinant in the development of oropharyngeal cancers (OPC) [134]. It is estimated that nearly 70% of OPC cases are HPV positive, with a substantial proportion of these cases occurring in the tonsils [135]. Additionally, approximately 85% of HPV-positive OPC cases are infected with high-risk variants, such as HPV 16 or HPV 18 [136]. These HPV-positive OPCs have been found to have distinct clinical characteristics when compared to HPV-negative tumours, particularly with respect to treatment response and overall survival rates [137, 138].

HPV-related OPCs are a rapidly growing global health concern, with increasing incidence rates reported in both developed and developing countries. In some regions, the incidence of HPV-positive OPC has now surpassed that of HPV-negative OPC [135]. Studies conducted the United States and Europe have demonstrated a sharp rise in the incidence of HPV-positive OPC, particularly in young adults. In the United States, the incidence of HPV-positive OPC has increased by 225% among young men in the last two decades [139]. In Europe, a similar trend has been observed, with incidence rates of HPV-positive OPC increasing by approximately 60% among young men in the last 10 years [140]. Likewise in Australia, research indicates a notable rise in HPV-positive OPC. A recent study in Australia found that the incidence of HPV-positive OPC amongst Australian men has increased by 1.5 times between 1982 and 2017, aligning with trends observed in other developed nations [280].

The trend of increasing HPV-related OPC is not limited to developed countries. In developing countries, the incidence of HPV-related OPC is rising and is expected to continue to increase [141]. In low- and middle-income countries within both Asia and Africa, the incidence of HPV-positive OPC has been increasing at a faster rate than in developed countries, with HPV 16 and 18 being the most common high-risk types identified [141].

High-risk strains of human papillomaviruses code for several oncogenes, in particular, E6

and E7. Under normal conditions, E6 and E7 are expressed at low levels and are thought to function by creating conditions in the infected oral keratinocytes that favour replication of the virus and prevent apoptosis of host cells [50]. At higher levels, these two oncoproteins have major effects on a variety of cellular functions that may lead to uncontrolled growth [142]. E6 is best known for its ability to bind to and mediate the degradation of the tumour suppressor p53 [143] and other targets involved in cellular apoptotic pathways [144]. As a consequence of these interactions, cells expressing E6 are much less likely to undergo apoptosis. E7 is known for its ability to bind to and inactivate the tumour suppressor Rb protein, disrupting its ability to regulate E2F transcription factors, resulting in disrupted cell cycle regulation [144].

The detection of HPV is a critical component in the diagnosis and management of HPV-related OPC. There are several techniques available for HPV detection, including: polymerase chain reaction (PCR)-based methods; hybrid capture (HC) assays; in situ hybridization (ISH); and p16INK4a (p16) overexpression detection via immunohistochemistry (IHC) [145, 146]. p16 detection is the most used method in the diagnosis of HPV-related OPC [147]. While p16 overexpression is a marker of HPV-associated malignancy, the interpretation of p16 results can be subjective and may result in inter-observer variability [148]. In addition, using the p16 detection method may produce false positive results, as p16 overexpression can occur due to other causes besides HPV infection [149]. This can result in the detection of p16 in tissue samples that are not actually infected with HPV. Moreover, p16 overexpression is a late event in HPV-associated carcinogenesis, meaning that it may not be present in early-stage cancers [46]. These limitations highlight the importance of using additional methods, such as PCR, to confirm the presence of HPV.

PCR methods have been widely used for the detection of HPV16 [54, 150-155]. However, the major problem with the PCR approach is that the detection of viral DNA does not indicate an active infection. The virus may be dormant, and patients, even though they test positive for HPV, may not go on to develop cancer [155]. Despite the various methods available for HPV detection, there is a lack of RNA-based HPV testing which can indicate an active infection. Furthermore, most of these tests require a tissue biopsy which may limit the scope of testing.

There has been an effort to evaluate saliva as a liquid biopsy for diagnosing certain diseases

[156-159]. Saliva is ideal as it is a water-based fluid containing trace amounts of inorganic elements and heterogenous populations of biological particles. It has been termed the “mirror to the body”, as it reflects local and systemic conditions [69]. Given this notion, it is plausible that saliva contains RNA species released by head and neck cancer cells or HPV16 within the oral cavity.

This study involved the development and evaluation of a probe-based multiplexing quantitative PCR (qPCR) technique to identify viable HPV16 RNA in the saliva of patients with OPC. The ability to detect active or replicating virus in the saliva of OPC patients would be a valuable clinical tool that could aid in directing appropriate treatment strategies for these individuals.

3.4 Materials and methods

Cell lines

The cell lines used in this study included: squamous cell carcinoma from the cervix, SiHa (HPV16 positive); epidermoid carcinoma from the cervix, CaSki (HPV16 positive); adenocarcinoma of the cervix, HeLa (HPV18 positive); and ductal carcinoma of the mammary gland, MCF-7 (HPV negative). For this study, we utilised cervical cancer cell lines, including SiHa and CaSki (HPV16 positive), and HeLa (HPV18 positive), due to their well-documented infection status and extensive use in HPV research. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX^{TM} (Thermo Fisher Scientific) with 1% glutamine, 10% foetal calf serum (Thermo Fisher Scientific*)* in a 37°C incubator with humidified 5% CO2.

Tissue specimens

Tissue specimens were retrieved from patients treated for Squamous Cell Carcinoma (SCC) of the Oropharynx at Royal Prince Alfred Hospital, Sydney, between 2002 and 2006. The study was approved by the Research Ethics Committee at Royal Prince Alfred Hospital, Sydney, Australia (Protocol number X05-0270). Informed consent was obtained for the collection of fresh tissues. Immediately after surgical resection, tissues were snap frozen on dry ice and stored at -70°C. The histology of tissues was assessed by hospital pathologists. For this study, six fresh-frozen tissues samples were selected from p16 positive (n=4) and p16 negative (n=2) OPC specimens.

Saliva specimens

All HNC saliva samples were obtained from patients through informed written consent with approval by the ethics board at Royal Prince Alfred Hospital, Sydney, between 2018 and 2022 (Ethics: X19-0195 and 2019/ETH11588). From each patient, 2mL of unstimulated whole saliva was collected directly into sterile collection tubes, which were snap frozen on dry ice and stored at -20°C until use. Two types of collection kits were used: a non-commercial kit, consisting of sterile Eppendorf tubes, and a commercial kit, namely the DNA/RNA Shield SafeCollect Saliva Collection Kit (Zymo Research). For this proof-of-principle study, we collected saliva specimens from p16 positive (n=9) and p16 negative (n=5) HNC patients.

RNA Isolation from Cell Lines

A cell pellet containing 5×10^6 cultured tissue cells was homogenised by adding 1mL of RNAzol® RT (Molecular Research Center). The homogenate was incubated for five minutes after the addition of 0.4mL RNase-free water (Invitrogen, Thermo Fisher Scientific) for DNA, protein and polysaccharide precipitation, and centrifuged at 12, 000 x *g* for 10 minutes. The supernatant was then transferred to a fresh tube and 5mL 4-bromoanisole (Molecular Research Center) was added for RNA purification. The sample was incubated for three minutes and centrifuged at 12, 000 x *g* for 10 minutes. RNA was precipitated out by adding one volume isopropanol (Sigma Aldrich) to the supernatant. The sample was incubated overnight at -20°C and then centrifuged at 12, 000 x *g* for 10 minutes. The supernatant was then discarded, and the RNA pellet was washed twice with 75% ethanol (Sigma Aldrich) by centrifugation at 12,000 x *g* for 5 minutes. Lastly, the RNA pellet was resolubilised in 20mL of RNase-free water.

RNA isolation from Tissue

Briefly, 100mg of fresh frozen tissue was diced with a surgical blade, homogenised with a mortar and pestle, and rinsed with 1mL of RNAzol® RT. 0.4mL water was added to the sample and centrifuged at 12,000 x *g* for 10 minutes to precipitate the DNA and proteins. The sample was purified using BAN and centrifuged again at 12,000 x *g* for 10 minutes. RNA was precipitated using isopropanol according to the above protocol, and the sample was incubated at -20°C overnight. The RNA was then washed with 75% ethanol twice and the RNA pellet was resuspended in 20 µL of RNase-free water.

RNA isolation from Saliva

Our previously published protocol to isolate total RNA from serum [124] was modified and adapted to isolate total RNA from patient saliva samples . Following the collection of saliva samples from storage (-30°C), the sample was centrifuged at 1,600 x *g* for 15 minutes at 4°C to separate the cellular debris from the supernatant. The salivary supernatant was then aliquoted into lots of 400µL for processing. Samples were homogenised with 1.5mL Tri-Reagent RT-Liquid Samples (Molecular Research Centre) and 100mL 4-bromoanisole was added for phase separation. Samples were centrifuged at 12,000 x *g* for 20 minutes at 4°C in 1.5mL phase-lock gel tubes (5PRIME), as per the manufacturer's protocol.

After the sample was centrifuged, the uppermost clear aqueous phase containing RNA was decanted into a fresh DNA Eppendorf Lo-bind tube. 500µL isopropanol and 5µL Glycogen was added to the sample. The sample was then inverted and stored for incubation overnight at -20°C. Following incubation, the sample was centrifuged at 12,000 x *g* for 20 minutes at 4°C. Immediately after centrifugation, the supernatant was removed, and the remaining pellet was flash spun (centrifuged) at 16,000 x *g* for two minutes at 4°C. Any remaining liquid was removed from around the pellet and then the pellet was washed twice with 1mL of 70% ethanol and allowed to air dry prior to re-suspension in 20µL of RNase-free water. For a higher total RNA yield, samples from the same participant were pooled into the same 20µL of RNase-free water.

RNA Quantification and Quality Control

The re-suspended total RNA was quantitated using a Nanodrop™ 1000 3.7.1 UV-Vis Spectrophotometer (Thermo Fisher Scientific). The Nanodrop stage was cleaned with water and 70% ethanol. Following this, the instrument was blanked using 1µL of RNase free water. Using 1µL of sample, the absorbance spectra was measured, and the data obtained from the 260nm peak indicated the concentration of total RNA. Absorbance ratios at 280nm (A_{260}/ A_{280}) and 230nm (A_{260}/A_{230}) were noted for purity purposes. Accepted ratios for purity vary with downstream applications, however, typical A_{260}/ A_{280} ratios should be between 1.8 - 2.2, while requirements for A_{260}/A_{230} ratios are generally greater than 1.7.

cDNA Synthesis

cDNA synthesis was prepared using the High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific, according to Supplementary Table 1.3. Tubes were then placed in a thermocycler and run using the following conditions: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C and the sample was held at 4°C until collected.

Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR); Multiplexing HPV16 Oncogenes E6/E7

Following cDNA synthesis, samples were diluted 1:4, and RT-qPCR was performed using a 5µL reaction. This was prepared according to the Table 1.3 (supplementary material) for RT-qPCR. All qPCR reactions were conducted in triplicate using the StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, USA) with the recommended cycling conditions for the TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fischer Scientific,

USA). TaqMan assays were designed for the oncogenes *E6* and *E7*, using Primer3Plus (https://primer3plus.com/) based on sequences obtained from NCBI (https://www.ncbi.nlm.nih.gov/refseq/). In order to multiplex these two oncogenes, E6 was labelled with a VIC™ and E7 was labelled with a FAM™ probe.

Primer sequences as follows:

E6

Context sequence: TGGACAAGCAGAACCGGACAGAGCC

Probe (VIC): TCCGGTTCTGCTTGTCC

Forward sequence: GCTCAGAGGAGGAGGATGAAATAGA

Reverse sequence: GAGTCACACTTGCAACAAAAGGTT

E7

Context sequence: ACCCAGAAAGTTACCACAGTTATGC

Probe (FAM): ACAGAGCTGCAAACAA

Forward sequence: ACCCAGAAAGTTACCACAGTTATGC

Reverse sequence: TGCTTGCAGTACACACATTCTAAT

Data Analysis

qPCR data was imported into GraphPad Prism (Version 8.2.1) and Cq values were plotted against sample using column graphs that compare both singleplex and multiplex qPCR results. PCR efficiencies were determined using LinRegPCR (v 2021.2) and measured on a scale between 1.0 and 2.0, with 2.0 representing 100% efficiency. PCR efficiencies above 1.5 were determined to be acceptable. A two-sided t-test was used to determine whether a significant difference was observed between singleplex and multiplex PCR reactions (Cq values) and their PCR efficiencies ($p<0.05$).

3.5 Results

HPV16 singleplex and multiplex with HPV16 positive cell lines

The E6 and E7 primer/probe combination were firstly tested by singleplex and multiplex qPCR using the HPV16 positive cell lines, SiHa and Caski, and HPV16 negative cell lines, HeLa and MCF-7. Cq values were measured for two total RNA input amounts, 100ng and 200ng. Both E6 and E7 were detected in the HPV16 positive cell lines only (Figure 1A & B). Despite a 2-fold increase in RNA input, the expression levels of both probes were lower in SiHa cells, which was determined to be due to this cell line only having two viral insertions of HPV16 [160]. Nevertheless, 100ng of input RNA was found to be adequate to detect both E6 and E7. Furthermore, no significant difference in Cq value was observed between the singleplex and multiplex qPCR methods. PCR efficiencies for HPV16 positive results for both the E6 and E7 were calculated using LinRegPCR. All HPV16 positive samples remained above an efficiency of 1.5, and no significant difference was observed between the mean qPCR efficiency of the singleplex and multiplex of E6 and E7 mRNA in samples at both 100ng and 200ng (p value <0.05) (Figure 1C & D). Overall, multiplexed samples fared better than singleplex in terms of PCR efficiency, particularly for the E7 assay.

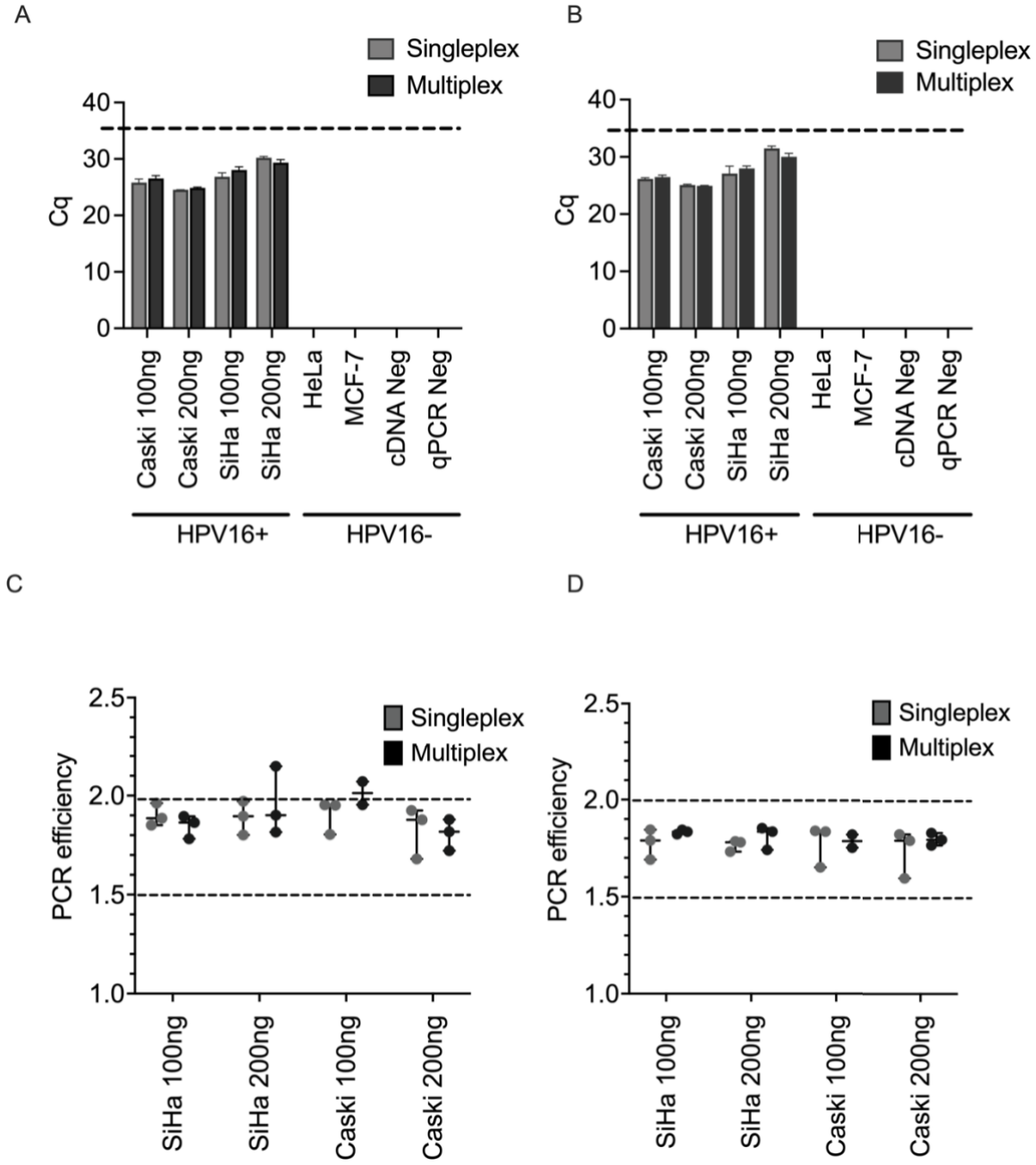

Figure 1. Detection of E6/E7 mRNA in HPV16 positive cell lines. A) E6 assay; B) E7 assay. HPV16 positive cell lines included SiHa and Caski, while HPV16 negative cell lines included HeLa (HPV18 positive), and MCF-7 (breast cancer cell line). Experimental controls included cDNA and PCR negatives. All reactions were completed in triplicate. PCR efficiencies for E6 and E7 mRNA assays in cell lines. C) E6 assay; D) E7 assay. 100% PCR efficiency is depicted at 2.0, with acceptable threshold of efficiency at 1.5.

Minimal RNA required for detection of HPV16

We then assessed the minimal amount of total RNA required for E6 (Figure 2A) and E7 (Figure 2B) detection. SiHa and Caski total RNA was diluted to a range of 50pg, 100pg, 500pg, 1ng, 2ng, 3ng and 4ng, and a Cq of 35 was designated to be a reasonable threshold for a positive result. The results showed that at 50pg of RNA input for both SiHa and Caski in the E6 assay, and for SiHa in the E7 assay, the Cq value was above 35 and thus deemed negative. All samples with RNA concentrations above 1ng were consistently below the Cq threshold. The minimal amount of RNA required was determined to be 100pg. No significant difference in Cq value was observed between the singleplex and multiplex methods ($p<0.05$). PCR efficiency was again calculated for the E6 and E7 assays in HPV positive samples using LinRegPCR (Figure 2C and Figure 2D). Although we noted some variation in the mean qPCR efficiencies, the majority of samples remained above the 1.5 threshold. In samples with minimal RNA input, singleplex reactions appeared to have better PCR efficiencies than multiplex reactions.

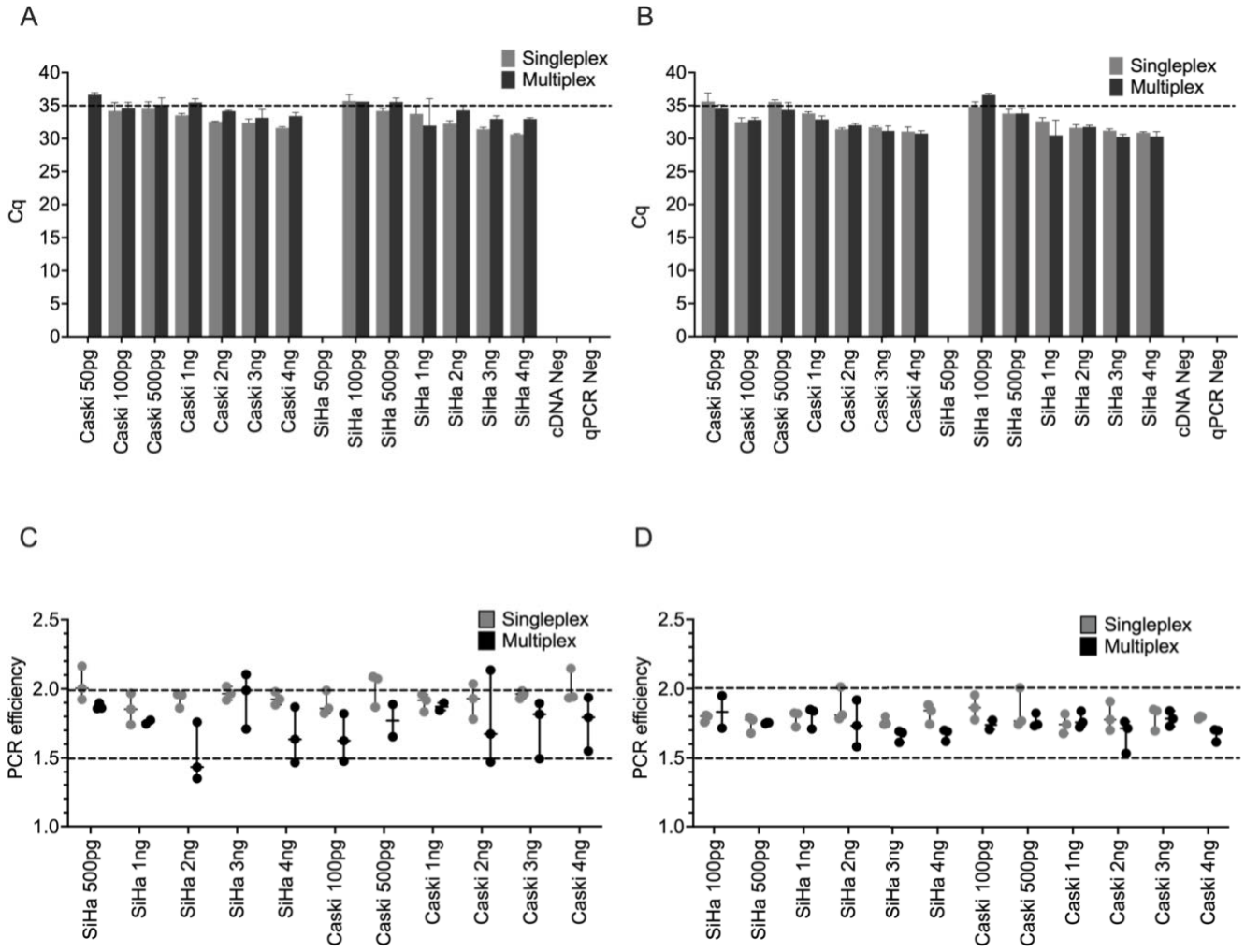

Figure 2. Total RNA input for E6 and E7 in assays in cell lines. A) E6 assay; B) E7 assay. Cq 35 was determined to be a reasonable threshold for a positive result. Experimental controls included cDNA and PCR negatives. All reactions were completed in triplicate. PCR efficiencies for C) E6 assay; D) E7 at different RNA inputs. 100% PCR efficiency is depicted at 2.0, with the acceptable threshold of efficiency at 1.5.

Single and multiplex detection of E6 and E7 using Patient Tissue

We then measured the expression of E6 and E7 in HPV16 positive and HPV16 negative patient tissue (Figure 3A, B, C & D). The isolated RNA was serially diluted to total inputs of 5ng, 10ng, 50ng, and 100ng for all patient samples. Both the E6 and E7 assays were successful in the detection of their respective targets in the single and multiplex format. As expected, when more RNA input was added, we observed a corresponding decrease in Cq values. E6 and E7 mRNA was not found in p16 negative patient tissue. From these results, 10ng of input RNA would ensure a positive result below the threshold, Cq 35. PCR efficiencies were also calculated using LinRegPCR (Figure 3E & F). Whilst the majority of samples remained above the established 1.5 threshold, we observed variations in the average qPCR efficiencies. In samples with minimal RNA input (i.e., less than 10ng of RNA), singleplex reactions obtained better PCR efficiencies than multiplex reactions. A sample input of 100ng of total RNA performed the best, with PCR efficiencies closest to 2.0.

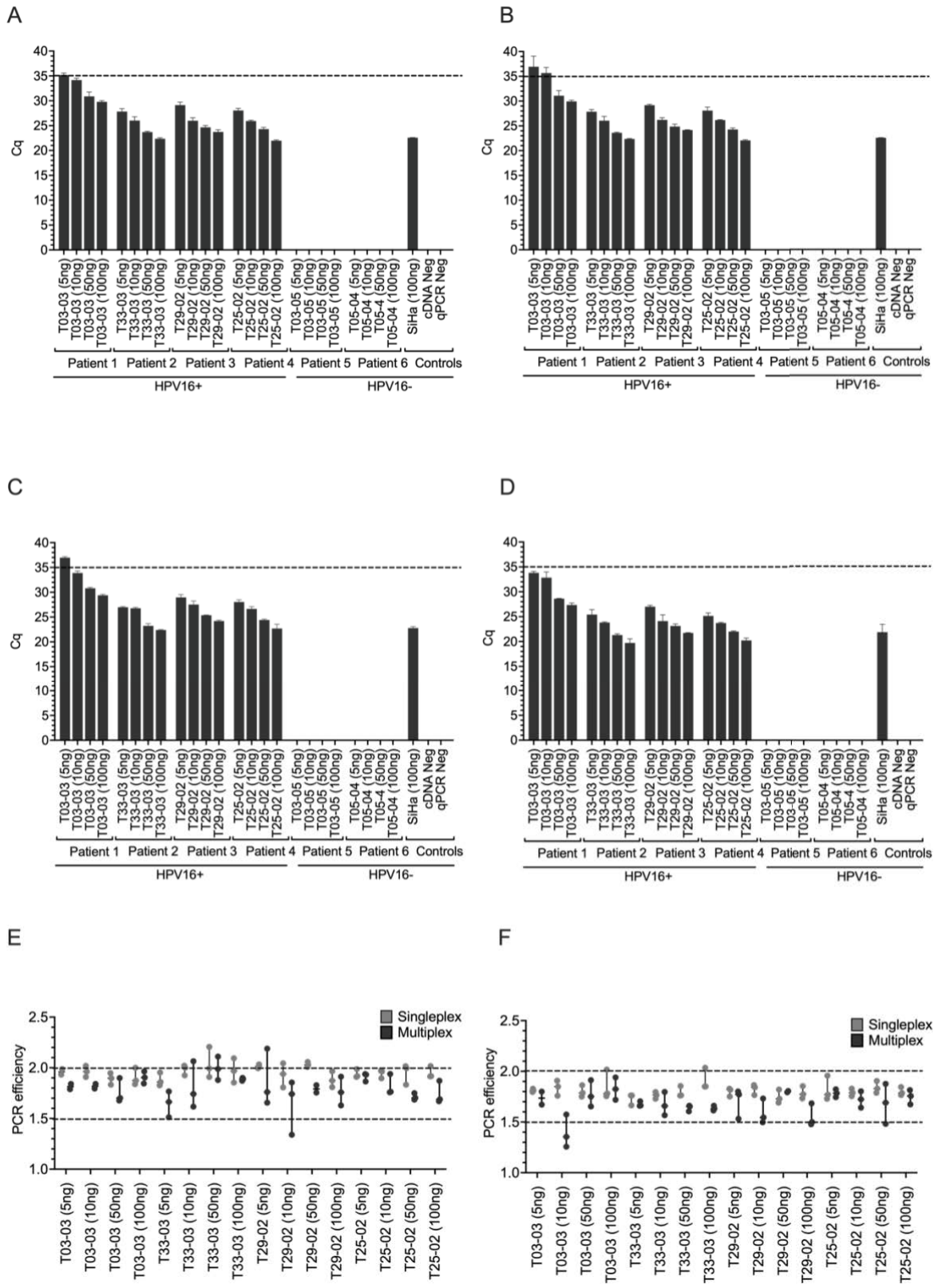

Figure 3. Multiplex and singleplex detection of E6 and E7 mRNA in patient tissue. A) Multiplex E6 assay; B) Multiplex E7 assay; C) Singleplex E6 assay; D) Singleplex E7 assay. Controls included SiHa (100ng RNA input) as a positive control, and cDNA and PCR negative samples. All reactions were completed in triplicate. E) PCR efficiencies for E6 and F) E7 mRNA assays in patient tissue specimens.

Detection of viable HPV16 virus in patient saliva

The collection method for saliva is pivotal for any downstream testing. We decided to compare two standard approaches; 1) the collection of whole saliva with no additives, and 2) the collection of saliva using a commercial kit (containing additives). After collection, total RNA was isolated per our laboratory's published methodology [161]. Figure 4A depicts the RNA concentrations obtained when isolating total RNA from whole saliva. The mean total RNA concentration for the salivary supernatant using the non-commercial kit was 67ng, which was significantly lower in yield compared to the mean total RNA concentration for the salivary supernatant collected using the commercial kit at 1,775 ng. We note that the while the mean total RNA obtained from the commercial kit was much improved from the non-commercial kit, the total RNA concentrations still have a large variation, similar to what we observed in whole saliva total RNA isolations. RNA was isolated from 28 individuals using the non-commercial kit methodology and 18 individuals using the commercial kit.

We then determined if the RNA from these commercial kits would provide usable material for PCR amplification. As shown in Figure 4B, we were able to detect the following targets, 18S, Beta Actin (ACTB), p53 and Dicer1, with RNA collected from both methods. RNA input into the qPCR was standardised to 100ng. Cq data for the commercial and non-commercial kits were aggregated to understand how the methods compared. For 18S, the mean Cq value for the non-commercial kit was 20.0 and the mean Cq value for the commercial kit was 12.8. Similarly, mean Cq values for ACTB were 29.4 and 25.2 for non-commercial and commercial kits respectively. Cq values for p53 were similar for both kits with a mean Cq value of 30.9 for non-commercial and 31.0 for commercial. Mean Cq values for Dicer1 were 33.7 for non-commercial and 29.5 for commercial. Overall, the commercial kit performed better across the various gene targets.

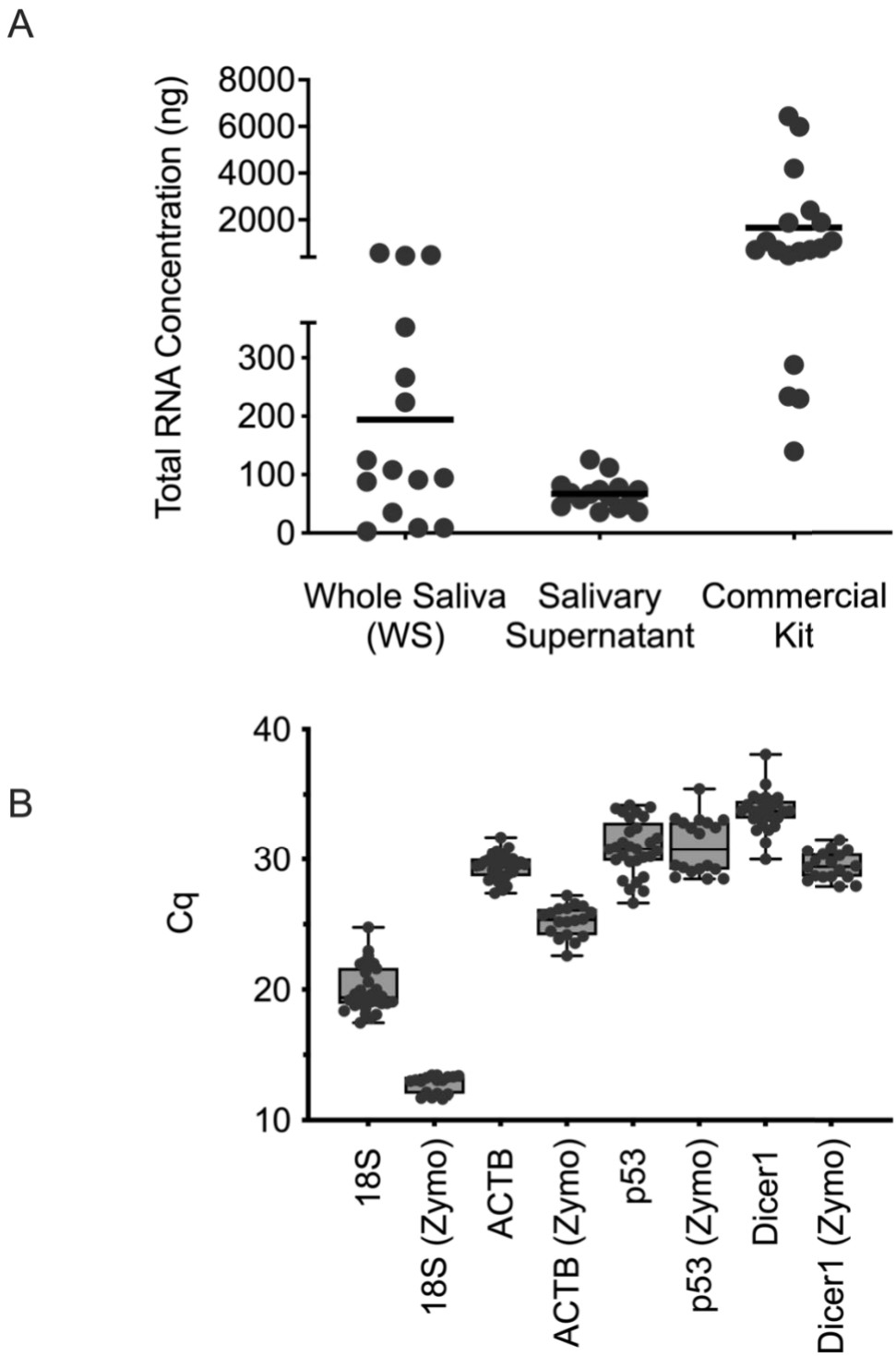

Figure 4. A) Comparison of total RNA concentration obtained from various saliva collection and processing methods. Whole saliva (n=12) and salivary supernatant (n=12) via the commercial Zymo saliva collection kit (n=12). B) Comparison of RNA expression of original saliva collection method vs commercial Zymo tubes. All reactions were completed in triplicate.

Multiplex qPCR of E6 and E7 mRNA in Patient Saliva

In the next step, we used isolated RNA from the collected saliva, to determine if a multiplex qPCR could be used to amplify and detect both E6 and E7 targets (Figure 5B & C). As a proof of principle, 100ng of total RNA was isolated from 1.2mL of saliva from eight HNSCC patient specimens, including three p16 positive and five p16 negative patients. Using a multiplex approach, both E6 and E7 were detected in p16 positive samples. The E6 assay (mean Cq 31.5) had slightly increased levels of expression compared to the E7 assay (mean Cq 34.4). As expected, the E6 and E7 targets were not found in the p16 negative patients. Beta 2 Microglobulin (B2M) was used as a both positive control and reference gene and was amplified and detected in all samples (Figure 5A).

PCR efficiencies of the HPV16 positive results for both the E6 and E7 assays were calculated using LinRegPCR (Figure 6A & B). Overall, both E6 and E7 performed with the same mean PCR efficiency of 1.8 across the two assays and methodologies. The E6 assay showed less variability in PCR efficiency than the E7 assay. All p16 positive patient saliva samples remained above the 1.5 efficiency threshold and there was no significant difference in mean qPCR efficiencies between the singleplex and multiplexing of E6 and E7 mRNA ($p<0.05$).

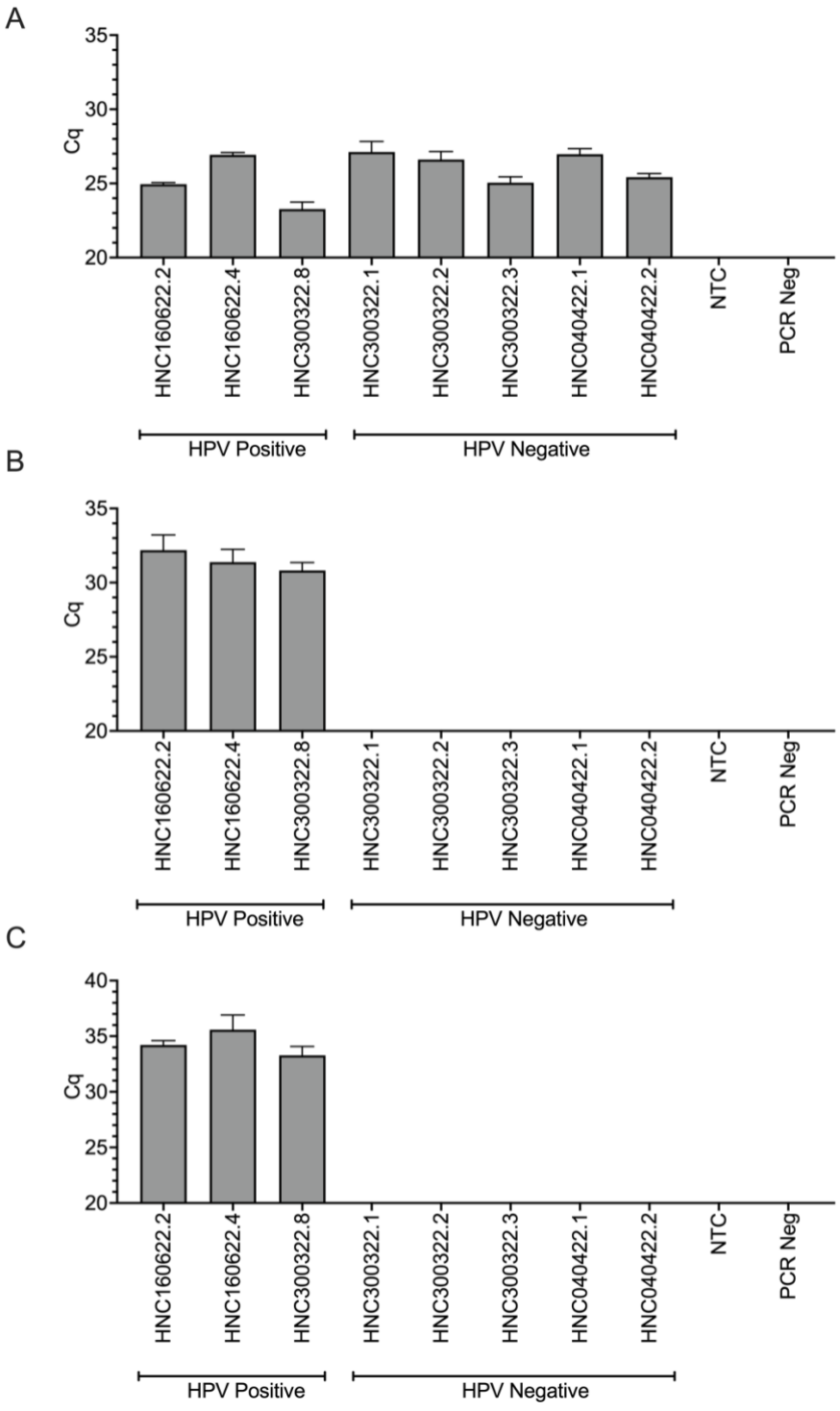

Figure 5. Proof-of-principle multiplex detection of E6 and E7 mRNA in saliva specimens. A) B2M assay; B) E6 assay; C) E7 assay. Using 1.2mL of saliva, RNA was isolated from the saliva of eight HNSCC patients (n=3 p16 positive; n=5 p16 negative). Beta 2 Microglobulin (B2M) was used as a positive control and reference gene to ensure expression observed in the E6 and E7 assays was positive and accurate. Negative experimental controls included cDNA (NTC) and PCR negative reactions. All reactions were completed in triplicate.

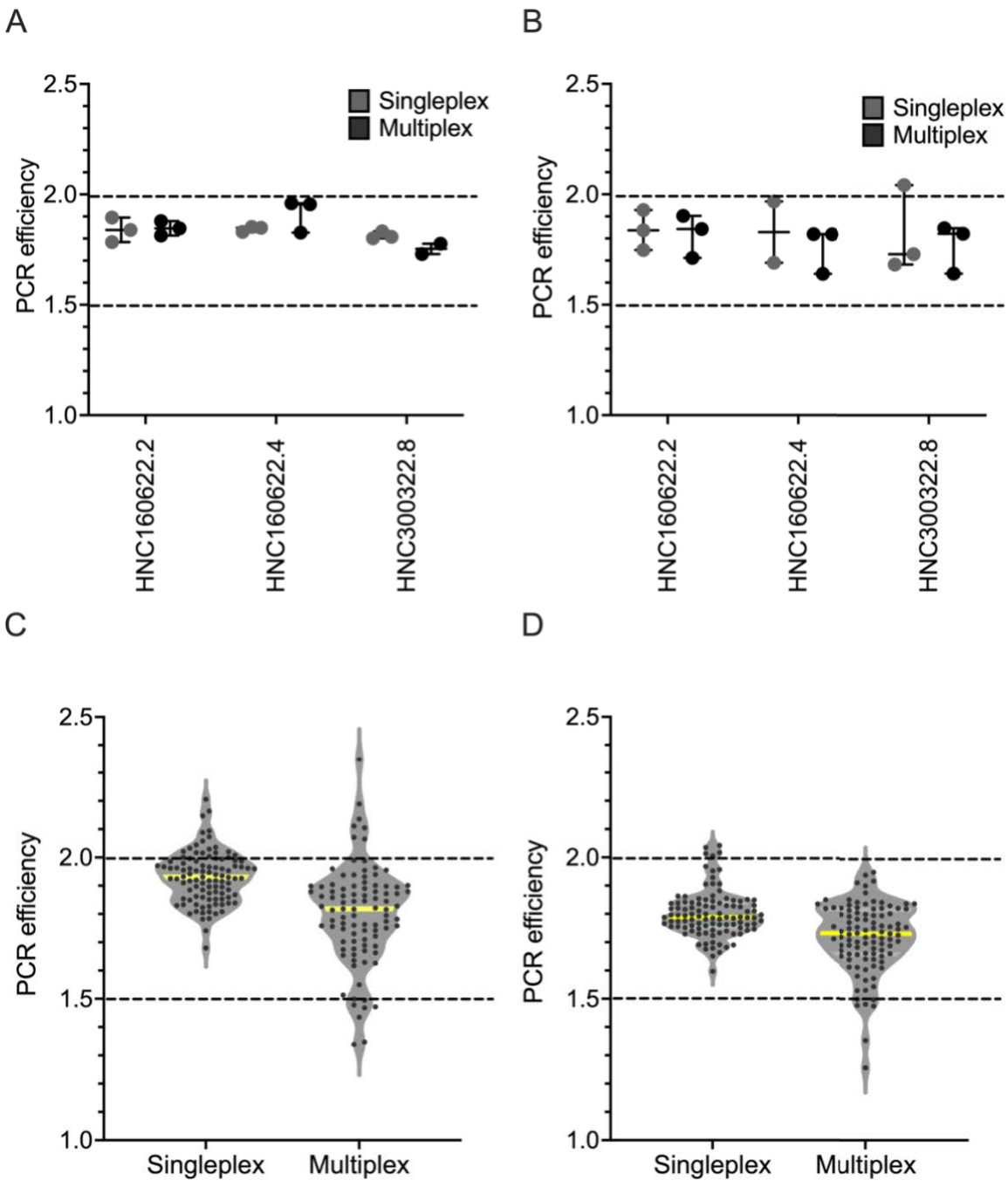

Figure 6. PCR Efficiencies for E6 and E7 in salivary specimens. A) E6 assay; B) E7 assay. 100% efficiency is depicted at 2.0, with the acceptable threshold of efficiency at 1.5. C) E6 overall PCR efficiencies and D) E7 overall PCR efficiencies.

3.6 Discussion

From the results, we were able to successfully detect transcriptionally active E6 and E7 mRNA transcripts in HPV16 positive cell lines, SiHa and Caski with as low as 100pg of input RNA. Mean PCR efficiencies were approximately 1.8, which suggests optimal PCR amplification. This indicates that the PCR reactions are producing E6 and E7 amplicons with close to 100% efficiency. We then multiplexed the assays in p16 positive OPC patient tissues using various concentrations of input RNA. We detected transcriptionally active E6 and E7 mRNA in the p16 positive patient specimens only and increasing levels of mRNA expression was associated with increased RNA input. While some variability in PCR efficiency for these reactions was observed, particularly for samples with low input RNA (<10ng), mean PCR efficiencies remained above 1.7.

Patients with HPV-positive OPC commonly exhibit better treatment outcomes and overall survival rates compared to their HPV-negative OPC counterparts [162]. The identification of HPV in OPC can guide treatment selection by identifying patients who may respond more favourably to chemotherapy and radiation therapy. Furthermore, HPV detection in OPC patients serves as a vital diagnostic and monitoring tool, providing prognostic information for these patients [163].

The oncogenes E6 and E7, which are encoded by HPV, play a critical role in the development of HPV-associated cancer by disrupting the normal cell cycle and promoting uncontrolled cell growth. Thus, they are useful biomarkers for the detection of HPV and the identification of high-risk HPV-associated cancer [164, 165] [166]. In these patients, high-risk HPV-status is a well-proven, independent prognostic factor for survival [167-170].

High-risk HPV infection is also closely linked to the overexpression of p16 protein. Positivity for p16 is now integrated into the recent 8th edition of tumour-node-metastasis (TNM) classification by the Union for International Cancer Control (UICC), and p16-positive OPCs are substantially down-staged compared to p16-negative OPCs [150]. Although p16-IHC is highly sensitive for high-risk HPV infection, it is not an entirely specific surrogate marker [150], which results in false positives due the overexpression of p16 that is not linked to HPV infection. It is estimated that this problem affects approximately 5-10% of all OPCs [171-173].

Consequently, the use of p16-IHC or HPV-specific testing alone as a reliable means of determining HPV status has been called into question, with recent studies identifying a subgroup of patients with discordant p16 and HPV positivity [174]. Specifically, most of the discrepant cases reported to date are p16-positive but HPV DNA-PCR or DNA-ISH negative. In light of these findings, the College of American Pathologists (CAP) recommends additional HPV-specific testing at the discretion of the pathologist and/or treating clinician following p16 testing [175]. These developments underscore the need for greater scrutiny of testing methods and the importance of accurate HPV status determination in guiding clinical decision making.

It is for this reason that viral RNA expression has been suggested as the gold standard for a viable infection, meaning the virus is transcriptionally active. In this study, we sought to develop and optimise a method to non-invasively detect virally active high-risk HPV16 using a multiplex qRT-PCR in OPC patient saliva.

As expected, when testing the OPC saliva samples, transcriptionally active E6 and E7 mRNA was only found in the p16 patient saliva samples, confirming the specificity of the assays. The E6 assay performed slightly better than the E7 assay with a mean Cq value of 31.5, compared to 34.4 respectively. This reflects other studies [176, 177], where the E6 protein is thought to be more consistently expressed across different types of HPV and different stages of infection, and the expression of E7 is more varied. The mean PCR efficiency was 1.8 across both E6 and E7 assays and the singleplex and multiplex methods, indicating the salivary assays worked with high efficiency.

Saliva testing offers advantages over blood and tissue-based testing due to its non-invasiveness and ease of sample collection, allowing for a time- and cost-effective diagnosis. Multiplexing assays to include both E6 and E7 mRNA targets offers confirmation of active virus (viable infection) and may provide an alternative to the need for DNA-PCR or ISH tests. Furthermore, this methodology is scalable and well-suited for high-throughput screening, making it an attractive option for widespread screening programs.

Several studies have sought to use saliva for oral cancer detection but very few studies to date have used RNA to detect viable infections [21, 178-184]. In one Australian study, it was demonstrated that saliva rinses could be used to detect key HPV-DNA oncogenic targets with 92.9% sensitivity. They found that 39 of 42 oral rinses from p16 positive patients had detectable HPV16-DNA [179]. Another study using oral rinse from 110 patients employed nested PCR to detect low copy numbers showed a sensitivity rate of 75% [182].

Even though p16 detection in tumour tissue is the conventional method for HPV16 testing, studies have demonstrated that the detection rates for HPV16 are comparable between tissue and oral rinses, with the prevalence of HPV DNA similar in tumour tissue (59.2%) and oral rinses (53.2%). Furthermore, antibodies specific to HPV16 E6 and E7 were present in serum at a similar rate of 51.4%. Although the sensitivity rates were low, it suggests that HPV detection in oral rinses may be comparable with the gold standard method of p16 testing in tumour tissues [181].

We note that digital PCR (ddPCR) was previously used to detect HPV16 RNA in oropharyngeal swabs. It was demonstrated that the sensitivity and specificity of ddPCR in detecting the biomarker p16 were 92% and 98%, respectively. It also showed that ddPCR was able to detect E6 and E7 mRNAs with much higher sensitivity than conventional RT-qPCR. This was achieved using 20-50-fold less RNA than what is required for conventional RT-qPCR [184].

The main caveat with our approach is that the qPCR method only detects RNA, however there is the possibility of false negatives. That is, the virus is dormant and not transcriptional active. It may be possible to use this salivary qPCR as a companion diagnostic to p16 staining and a dual positive result may be more clinically informative than p16 staining alone. The future development of this assay to include other HPV16 targets may resolve these caveats. One possible approach is to identify HPV16 genes associated with viral dormancy and include these targets along with E6 and E7. The E2 gene is frequently overexpressed during viral latency and a key regulatory of both E6 and E7 [185]. The triumvirate of E2, E6, and E7 targets might be able to discern between viable and latent viral infection. Another strategy is to detect both the presence of viral DNA and RNA in the same qPCR assay. Other viral DNA targets could include the L1 and L2 genes which are highly

conserved [186, 187] or non-transcribe regions of the HPV16 genome. The latter would be an ideal qPCR target as it is not transcribed and only primers designed for this region would bind and amplify the DNA sequence.

We also acknowledge that the sample size used for the salivary testing is limited and a larger cohort will be required to further assess the sensitivity and specificity of this salivary qPCR method. An additional hurdle in utilizing salivary samples is the absence of universally recognized standards for the collection and handling of such specimens. A consistent collection protocol and a reliable approach for extracting genomic material must be established to address this issue [161]. Presently, only a limited number of techniques are available that can extract both DNA and RNA from a single salivary sample [188, 189].

In conclusion, our findings underscore the potential of salivary HPV16 testing as a viable component of liquid biopsy protocols, particularly given the successful detection of transcriptionally active E6 and E7 mRNA transcripts in both HPV16 positive cell lines and p16 positive patient specimens. Despite the challenges associated with sample variability and the need for standardization in sample collection and processing, the promising PCR efficiencies achieved suggest that this approach could become a critical tool for non-invasive HNC diagnosis. As we continue to refine the techniques and expand the range of detectable RNA/DNA targets, the development of a robust, standardized qPCR liquid assay for HPV detection appears increasingly feasible. These advancements could significantly enhance the diagnostic accuracy and timeliness for patients with HNC, ultimately improving patient outcomes through earlier and more precise treatment interventions..

Chapter 4

Trends in Head and Neck Cancer Incidence in Ho Chi Minh City between 1996 - 2015

4.1 Copyright Information

This chapter is under review in the journal of ***Head and Neck***.
The text presented here is the current version of the manuscript. Numbering of sections, style of referencing, number of tables and figures are altered to align with the formatting of the thesis.

4.2 Author Contribution

Authors: Fiona Deutsch, Dung X Pham, Nguyen Dinh Hien, Tuan, V. Nguyen, and Nham Tran.

Fiona Deutsch (graduate student) is the shared first author of this Research article. Her contribution to this manuscript is demonstrated by the following roles/ tasks: researched the field, undertook a literature review, undertook bioinformatics and analysis of data, prepared the tables and figures, and was actively involved in the initial drafting, editing and review process.

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4.3 Abstract

The burden of head and neck cancer in Vietnam has not been documented. This study sought to estimate the incidence of head and neck cancer, and its subtypes, in Ho Chi Minh City, the largest economic centre of Vietnam, from 1996 to 2015. This was a population-based study using Ho Chi Minh City Cancer Registry as a source of data (coverage period: 1996-2015). The Registry adopted the International Classification of Disease for Oncology, 3rd Edition for the classification of primary sites and morphology, and guidelines from the International Agency for Research on Cancer and the International Association of Cancer Registries. Using the population statistics from census data of Ho Chi Minh City, the point incidence for Head and Neck cancer for a 5-year period was estimated for its four subtypes: oropharyngeal cancer, oral cancer, nasopharyngeal cancer, and larynx/pharynx cancer. Based on the national population, we calculated incidence and the age-standardised rate (ASR) of head and neck cancer and the subtypes during the 1996 – 2015 period. Our analysis found that Head and Neck cancers increased in Ho Chi Minh City between 1996 and 2015. Of the four subtypes, Vietnamese people are affected by nasopharyngeal and oral cancers more than the other subtypes.

4.4 Introduction

Head and neck cancers (HNC) represent more than 550,000 cases per year globally and are the sixth most common cancer type [12]. They are a heterogenous group of cancers that affect the areas of the upper aero-digestive tract. Its major subtypes include oral cancer, oropharyngeal cancer, nasopharyngeal cancer, notably prevalent in southeast Asia due to its strong association with Epstein-Barr virus (EBV), and cancers of the laryngo-pharyngeal area. The complex aetiology of head and neck cancer comprises of both intrinsic and extrinsic factors. In addition to the two major risk factors associated with the development of head and neck cancer [190], particularly habitual tobacco and alcohol consumption, it is now broadly accepted that viral infections (e.g., Human Papillomavirus, HPV) are also critical in this cancer's pathogenesis [17].

There is a geographic disparity in the distribution of head and neck cancers, most notably, that head and neck cancer is the most common cancer in economically disadvantaged countries [191], particularly observed in south and southeast Asia [192]. Many of these occurrences are attributed to the use of betel quid, especially prevalent in regions like Hunan Province in China and other parts of southeast Asia [193]. In western populations, the decrease in combustible tobacco use and increased HPV prevalence have allowed an epidemiologic shift that has heightened the role of high-risk HPV strains in HNC pathology [194]. This subset of the disease is seen more often in younger patients and most commonly presenting in the oral cavity, tongue, and oropharynx.

The lifetime risk of developing HNC is approximately 1 in 100 in developed countries, although these statistics are skewed towards men and people aged over 60 [195]. The cost of HNC, including diagnosis, treatment, and ongoing care, is significant. In the United States, the annual economic burden is estimated to exceed $3.2 billion [196].

Shifting focus to southeast Asia, this region witnesses a higher prevalence of HNC due to risk factors like high tobacco usage, alcohol consumption, and betel quid chewing. The lifetime risk of developing HNC in this region is higher than the global average, although precise data is lacking [197, 198]. The economic burden is also substantial. For instance, in Thailand, the annual cost of oral cancer treatment, a subset of HNC, is estimated at over US$20 million [199, 200].

In Vietnam, the specific statistics for HNC are not clearly defined due to limited research and data availability. However, considering the prevalent risk factors similar to those in other southeast Asian countries, it is likely that the incidence, lifetime risk, and economic burden of HNC are high.

Given the financial constraints of the country's healthcare system, this creates a pressing issue that demands urgent attention.

The first step to addressing the burden of HNC in southeast Asia is to analyse surveillance data for trends in the incidence of HNC. In the past few decades, rapid socioeconomic development has allowed improved control of communicable diseases in Southeast Asia, which resulted in the emergence of cancer as the leading cause of death in these countries.

Until now, there has been no systematic documentation of the incidence of head and neck cancer in Vietnam over the past 30 years. During this period, Vietnam has experienced rapid and comprehensive sociocultural change, as a result of the economic transition. This economic transition has impacted traditional and cultural behaviours and has contributed to various public health outcomes.

Vietnam has very high smoking rates among its population; in 2015, the daily smoking rate among men was 38.7% (estimated from the 2015 Global Adult Tobacco Survey) [201]. Furthermore, there are a large proportion of people exposed to second-hand smoke. In 2016, 63% of the population reported living in households where tobacco is consumed [202]. Alcohol consumption for adults almost doubled from 2003–2005 to 2008–2010, increasing from 3.8 to 6.6 litres of pure alcohol. This rate is slightly above the global rate of per capita alcohol consumption per annum, which is estimated at 6.2 litres [203, 204]. Understanding the impact of HPV16 on HNC in Vietnam presents a challenge due to the scarcity of research investigating its prevalence. Despite this, one notable study by the International Agency for Cancer Research (IACR) has provided some insight, suggesting a seropositivity rate of 21% for HPV16 in Ho Chi Minh City [205].

Given the rapid economic and social changes in Vietnam and the observed increases in risk factors for HNC, it would be timely to report on the incidence of this disease. In our study, we sought to estimate the incidence of head and neck cancer in Ho Chi Minh City, a population of 9 million inhabitants and the largest city in Vietnam. Our aims were threefold; Firstly, we looked to quantitate the incidence of head and neck cancer and its four main subtypes in Ho Chi Minh City between 1996-2015. Secondly, we analysed age standardised rates of head and neck cancer; and third, we examined defining points of incidence through joinpoint regression analysis.

4.5 Methods

Ho Chi Minh City Cancer Registry

The anonymised data for this study was extracted from the Ho Chi Minh City Cancer Registry [206-209]. The Registry was established in 1990 to document all diagnosed cancer cases in the City. Cancer patients admitted to any hospital in the city were ascertained and checked for possible duplication. The coverage period was from January 1, 1996, to December 31, 2015. This study was restricted to people who were identified as residents of Ho Chi Minh city on their patient records. We focused on Ho Chi Minh City, as (i) it is the largest centre of commerce in the country, with a population of 8.2 million (2014 statistics); (ii) the ascertainment and documentation of cancers in the City is more complete than any other provinces in the country; and (ii) the City offers an opportunistic setting for studying the burden of cancers in a transitional population. The study was approved by the Ethics Committee of the Oncology Hospital of Ho Chi Minh City.

The Ho Chi Minh City Cancer Registry adopted the International Classification of Diseases for Oncology, 3rd Edition (ICD0-3) for the classification of primary sites of morphology and guidelines from the International Agency for Research on Cancer and the International Association of Cancer Registries. Based on the ICDP-3, we identified head and neck cancer cases (including its four subtypes) from 1 January 1996 to 31 December 2015 inclusive. The identification was further ascertained by tumour site code, morphology code and behaviour type.

Data from all registries was collected and assessed based on guidelines from the International Agency for Research on Cancer and the International Association of Cancer Registries, adapted to a low and middle-income context. Data was validated through clinical records, coded, and verified according to guidelines.

Calculating ASR

Age and gender population statistics were obtained from census data managed by the General Statistics Office (GSO) of Ho Chi Minh City. Population statistics were available for 1999, 2004, 2009 and 2014. Age and gender population statistics in 1999 for Vietnam were obtained from the Bureau of Statistics of Vietnam.

Using the population statistics of Ho Chi Minh City, we computed the point incidence rate of Head and Neck cancer (per 100,000 population) for each 5-year interval: 1996-2000, 2001- 2005, 2006-2010, 2011-2015 inclusive. The reason for aggregating 5-year data was to improve the stability of statistical estimates. We used the direct method of standardisation to calculate the age-standardised rate (ASR), by applying the age-specific rates observed in a period to the national population in 1999. In this approach, the ASR can be through of as a weighted average rate, with the weights being the proportion of the national population in each age group.

Logistic joinpoint regression model

Using the United States National Cancer Institute SEER*Stat software (https://seer.cancer.gov/seerstat/), we employed a logistic joinpoint regression model to identify temporal changes in the incidence of Head and Neck cancer incidence in the Vietnamese population between 1996 and 2015. Data was first formatted in SEER*Prep using the Global Incidence File Format. Age-and-gender population statistics were obtained from census data managed by the General Statistics Office (GSO) of Ho Chi Minh City. Population statistics were available for 1999, 2004, 2009, and 2014. Age-and-gender population statistics in 1999 for Vietnam were obtained from the Bureau of Statistics of Vietnam. Cases were restricted to the head and neck cancer site codes according to the International Classification of Diseases for Oncology, Third Edition (ICD-O-3).

Using the Joinpoint Regression Program within SEER*Stat, we conducted a time-trend analysis to identify the years when significant changes occurred in the incidence rates. We used the Bayesian Information Criterion (BIC) to select the best-fitting joinpoint model, which detects the number and location of joinpoints (i.e., years where the rate of incidence changes) over the study period. We allowed for a maximum of 2 points in the model. We also calculated the annual percentage change (APC) and the average annual percentage change (AAPC) with 95% confidence intervals (CIs) to quantify the magnitude and direction of trends. All statistical analyses were performed using SEER*Stat version 8.3.8 and R version 4.0.2. A p-value less than 0.05 was considered statistically significant.

4.6 Results

Recorded HNC cases in Ho Chi Minh City 1996-2015

Between January 1, 1996, and December 31, 2015, 8,974 new cases of Head and Neck cancer had been registered in the Registry (Figure 1 and Supplementary Table 1). Based on the clinical grade (n=6,523 male, n=2,451 female), the majority of cases were at Grade II for males, and Grade I for females. Overall, 29% of head and neck cancer cases were classified as Grade II, which was the most prevalent grading. The majority of cases were diagnosed through histology of primary cancer (88.66%), with 64.5% and 24.16% of cases detected using this method for male and females, respectively.

By subtype, Nasopharynx cancer was the most common cancer in males (23.08%), whereas in females, cancer of the oral cavity was the prevalent cancer at 12.31% (Supplementary Table 2). 14.53% of nasopharynx cases were diagnosed at grade IV, whereas the majority of cases were diagnosed as grade I for larynx and pharynx cancers and cancers of the oral cavity. For oropharynx cases, the majority were graded at grade II. For all cancer subtypes, the basis of diagnosis was overwhelmingly by histology of primary cancer 18.56%, 28.83%, 30.63% and 10.63% for larynx/pharynx, nasopharynx, oral cavity, and oropharynx cases, respectively.

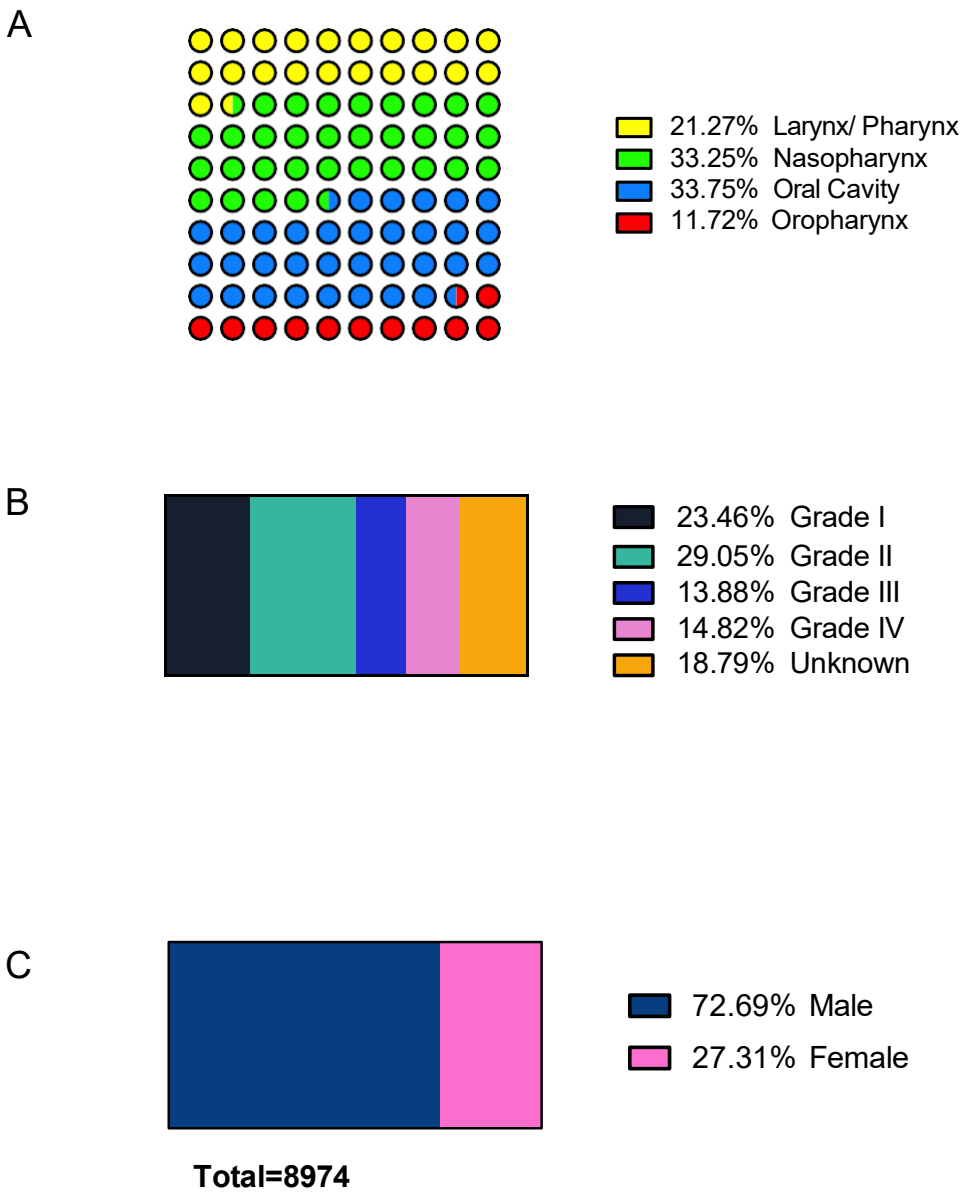


Figure 1. Clinical characteristics of Ho Chi Minh City Cancer Registry, Head and Neck cohort (n=8,974). A) Cancer subtypes include laryngeal/ pharyngeal cancer, nasopharyngeal cancer, oral cavity and lip cancer, oropharyngeal cancers; B) Grade of cancer at diagnosis indicating abnormality of cancer cells and aggression of cancer; C) Patient sex as recorded in Cancer Registry.

Incidence rates of HNC in Ho Chi Minh City 1996-2015

Overall, all four head and neck cancer subtypes saw increases in incidence over the 20 years examined (Figure 2). Nasopharyngeal and oral cavity cases were the most prominent cancer subtypes diagnosed in Ho Chi Min City between 1996 and 2015. Laryngeal and pharyngeal cancers were also increasing, realising its highest level of incidence in 2014. Oropharyngeal cancers had the lowest incidence, however also increased steadily over the 20 years.

The median age of diagnosis for all head and neck cancers was 56 for males and 57 for females, respectively (Table 1). Males were diagnosed at a lower median age than females for laryngeal/ pharyngeal cancers (61 years of age) and oral cavity cancers (56 years of age), six and 11 years prior to females, respectively (67 years of age for both cancer types in females). In contrast, women were diagnosed slightly younger than males with nasopharyngeal and oropharyngeal cancers; 55 years and 47 years respectively for females and 56 years and 50 years respectively for males (one year earlier in oropharyngeal cancers and 3 years prior for nasopharyngeal cancers).

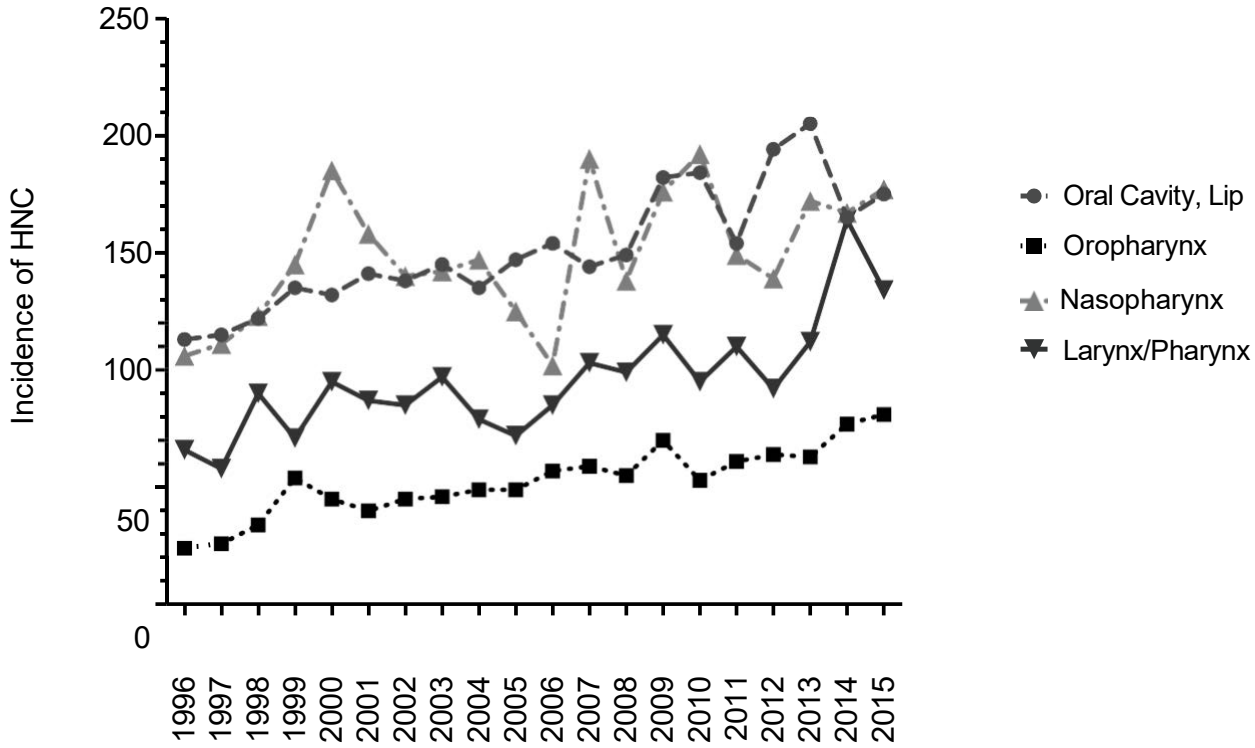


Figure 2. Point incidence of four head and neck cancer subtypes in Ho Chi Minh City between 1996 and 2015. Oral cavity cancers and nasopharyngeal cancers were the most prominent cancer type, followed by laryngeal/ pharyngeal cancers and oropharyngeal cancers.

Table 1. Median age of diagnosis for males and females residing in Ho Chi Minh City Vietnam by subtype (years of age). Males were diagnosed earlier than females for laryngeal/pharyngeal and oral cavity cancers, whereas females were diagnosed earlier than males for oropharyngeal cancers and nasopharyngeal cancers.

	Laryngeal/ pharyngeal	Oral cavity	Oropharyngeal	Nasopharyngeal	Head and Neck (all)
Male	61	56	56	50	56
Female	67	67	55	47	57

Overall, head and neck cancers increased between 1996 and 2015 for both males and females in Ho Chi Minh City. Five-year examination of incidence by age group saw both males and females between 40 and 69 years of age increasing, with the 50-59 age group highly representative in both sexes (Figure 3, A and B). There was a change in distribution of incidence in both males and females between the periods of 1996 and 2000, to 2011 and 2015. In the 1996 period, 25.59% of cases of head and neck cancer were in males aged between 60-69. In the 5-year period to 2015 to 35.39% of cases were made of up males aged 50-59. Likewise, in females with head and neck cancers, 25.2% of cases diagnosed between 1996 and 2000 were in females aged 70-79. In 2011 to 2015, 25.53% of cases were seen in females aged 50-59 (Supplementary Table 3A).

Nasopharynx cases remained consistently prevalent in males and females aged between 40-59 (Supplementary Figure 1, A and B). Nasopharyngeal cancers peaked in the 5-year period between 2006 and 2010 in both males and females in the 40-49 age group, representing 31% of nasopharyngeal cases in males and 33% in females for that time period (Supplementary Table 3B).

Oropharyngeal cancers affected males more than females in Ho Chi Minh City (n=2,071 for males; n=913 for females), with males in the 50-59 age group affected substantially more than other age groups (Supplementary Figure 1, C and D). In the period between 1996 and 2000, 27.2% of males with oropharyngeal cancers were diagnosed between 60-69 years old (Supplementary Table 3C). In 2011 to 2015, 41% were diagnosed in the 50-59 age range. In contrast, for females diagnosed with oropharyngeal cancers in 1996 to 2000, 18.97% of cases were diagnosed in the 40-49 age range. In 2011 to 2015, 29.41% of cases were diagnosed in the 60-69 age range.

Pharyngeal and laryngeal cancer incidence increased considerably in males and decreased in females between 1996 and 2015 (Supplementary Figure 1, E and F). Between 2001 and 2015, a steep increase was observed for males aged between 50-59. In the 2001 period, this age group represented 20% of pharyngeal/ laryngeal cases. By the 2011 to 2015 period, this age group represented 38% of these cases. In contrast, there were only 153 cases of laryngeal/pharyngeal cancers in females over the 20-year period examined (n=1,756 in males), which saw a slight decrease in cases between 1996 and 2015 (Supplementary Table 3D).

Similar to pharyngeal/ laryngeal cancers, incidence in the cancers of the oral cavity increased considerably in males and decreased in females between 1996 and 2015 (Supplementary Figure 1 G and H). Between 1996 and 2000, 23.9% of cases for males were diagnosed in the 70-79 age range

(Supplementary Table 3E). This age range lowered between 2011 and 2015, with the majority cases (34.08%) cases seen in the 50-59 age range. Similarly, in females, 37.12% of cases were diagnosed for patients between 70-79 in 1996 to 2000, however by 2011 to 2015, 27.61% of cases were seen in the 50-59 age range.

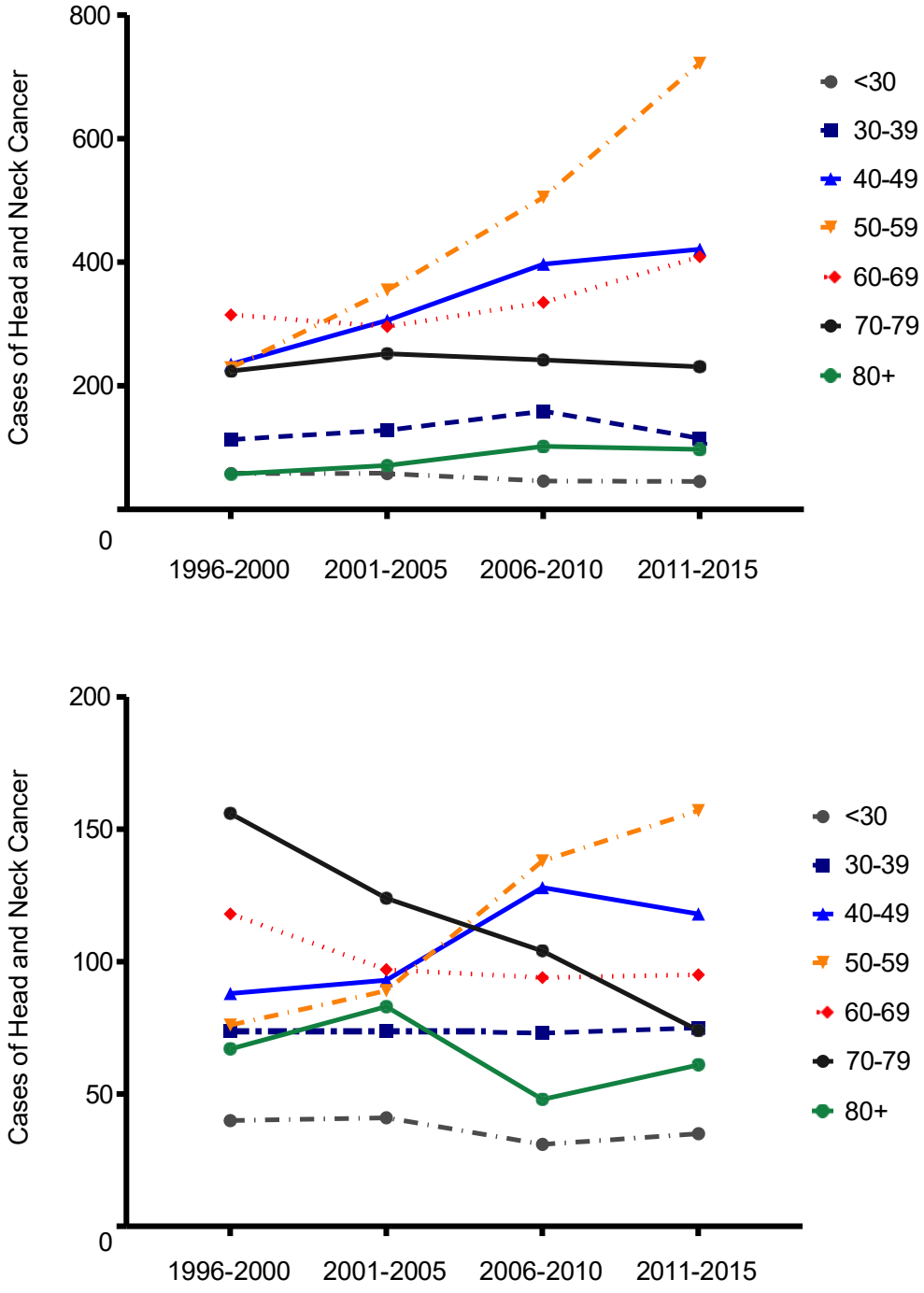


Figure 3. Five-year incidence of Head and Neck cancers by age group. A) Head and neck cancer incidence in males by age group. 50-59 age group increases substantially over 20 years examined; B) Head and Neck cancer incidence in females by age group. 50-59 age group increases over 20 year examined, whereas 70-79 age group steadily decreases in the same period.

The 5-year age-standardised incidence rate (ASR) for HNC

Overall, the age standardised rates for the head and neck subtypes remained somewhat consistent over the 20 years examined, except for nasopharynx, which decreased from 3.2 per 100,000 population to 2.7 per 100,000 (see Supplementary Tables 4, A-D). This is in addition to the average age of diagnosis increasing in this patient cohort (Supplementary Table 4, A-C). When comparing our findings on ASR and crude rates for head and neck patients in Ho Chi Minh City to GLOBOCAN statistics, head and neck cancer rates remain consistent for all subtypes, except again for Nasopharyngeal cancer, where the overall crude rate and ASR between 1996 and 2015 was 2.3 and 2.7 respectively. The crude rate and ASR for the world in 2020 was 1.7 and 1.5 respectively (per 100,000 population) (Supplementary Table 5). This correlates with Nasopharynx cases accounting for 33% of overall head and neck cancer cases in Ho Chi Minh city over the 20 years between 1996 and 2015.

If we now examine the HNC subtypes in more depth, the 5-year ASR for oral cancer was 3.5 cases per 100,000 population during the 1996 to 2000 period. This rate decreased slightly to 3.2 cases in 2011 to 2015. In males over the same time, ASR increased from 4.3 cases per 100,000 population to 5.5 per 100,000 population, while in females the ASR progressively decreased from 2.9 to 1.6 (Supplementary Table 5, A). For cancers of the oropharynx, overall, the ASR increased from the period 1996-2000 to 2011-2015 from 1 case per 100,000 population to 1.3 cases per 100,000 population (Supplementary Table 5, B). In males, this increased over the same period from 1.7 cases per 100,000 population to 2.2 cases per 100,000 population. In females, a smaller increase was seen of 0.5 cases per 100,000 population to 0.6 per 100,000 population over the same time period.

In Nasopharynx cases, the ASR decreased slightly from 3.2 cases per 100,000 population to 2.6 cases of the 20-year period (Supplementary Table 5, C). Males were overrepresented between 1996 and 2000, with an ASR of 5.1 cases per 100,000 population. Incidentally, the ASR decreased in the period 2011 to 2015 with an ASR of 4.4. This rate was still more frequent than females, which also saw a slight decrease in ASR from 1.8 to 1.3 in the same time period. Lastly, laryngeal, and pharyngeal cases slightly increased between 1996 and 2015 from 2.3 to 2.4 cases per 100,000 population (Supplementary Table 5, D). In males, the ASR increased from 5.0 cases per 100,000 population in 1996 – 2000 to 5.5 in 2011- 2015. For females, ASR decreased from 0.4 cases per 100,000 population to 0.2.

Joinpoint regression analysis

Joinpoint regression analysis did not identify breakpoints in any of the head and neck cancer subtypes throughout 1996 to 2015 (Supplementary Figure 1, A-D). In cancers of the oral cavity, age-adjusted rates increased over the 20 years, whereas females decreased in the same period (Supplementary Figure 1, A). Oropharyngeal and pharyngeal/ laryngeal age-adjusted rates increased in both males and females (Supplementary Figure 1, B; Supplementary Figure 1, D), whereas nasopharyngeal age-adjusted rates decreased slightly for both males and females (Supplementary Figure 1, C).

4.7 Discussion and Conclusion

This study represents a comprehensive 20-year analysis of Head and Neck Cancer (HNC) cases in Ho Chi Minh City, Vietnam. Over this period, 8,974 Vietnamese individuals were diagnosed with HNC with a disproportionate gender distribution. Specifically, males constituted 73% of these cases, significantly outnumbering female cases which made up the remaining 27%. The study examined four HNC subtypes, namely cancer of the oral cavity (34% of cases), nasopharyngeal cancers (33%), oropharyngeal (12%) and laryngeal/pharyngeal cancers (21%), demonstrating that HNC subtype prevalence in Vietnam is not dissimilar to that observed globally [210]. Intriguingly, a significant age gap was identified in Vietnam, with diagnosis typically occurring around 10 years earlier than global averages. There was also a decrease in the mean age of diagnosis over the 20-year period investigated, a stark contrast to increasing diagnosis age trends observed in Western countries [211].

Our findings are consistent with observations in other Asian countries and globally. Similar to Taiwan and the Philippines, oral cancer is a significant health burden in Vietnam, with more than 5,000 new cases every year in Taiwan [212] and a rate of 2 per 100,000 in the Philippines [213]. Nasopharyngeal cancer, the leading HNC subtype in China and Hong Kong, also accounted for a considerable proportion of Vietnamese cases [214, 215]. However, unlike Western countries, where oropharyngeal cases have sharply risen due to increasing human papillomavirus (HPV) infections [56], this subtype only accounted for a smaller fraction of HNC cases in Vietnam. Despite this, there has been a steady increase in the case numbers of oropharyngeal cancers in Vietnam over the 20-year period (Figure 2), and this may be due to HPV type-16, the most prevalence strain of the HPV virus globally [216, 217]. However, we do not have accurate rates for HPV-16 infections for this region, particularly for males, with previous research looking at HPV infection rates among Vietnamese woman [218, 219]. It was recently reported that HPV in Vietnamese males was common with high-risk HPV genotypes, although this study looked at penile cell samples [220].

Our study has also revealed notable disparities in the median age of diagnosis for various HNC subtypes in Ho Chi Minh City. Notably, for laryngeal and oral cancers, the median age of diagnosis was higher in females (67 years for both subtypes) than in males (61 and 56 years, respectively, as indicated in Table 1. Conversely, for oropharyngeal and nasopharyngeal cancers, males presented at a median age of 56 and 50 years, respectively, which was slightly older than their

female counterparts diagnosed at 55 and 47 years. Taken together, the median age of diagnosis for males was 56, a year younger than the median for females at 57.

In a global context, the median age of diagnosis for non-virally associated Head and Neck Squamous Cell Carcinoma (HNSCC) is typically 66 years [221]. Viral-associated HNCs, such as HPV-associated oropharyngeal cancer and Epstein-Barr virus-associated nasopharyngeal cancer, exhibit a lower median age of diagnosis, around 53 and 50 years, respectively [222]. Data specific to median age of diagnosis in Asia remains scant, but strikingly, there exists a 10-year disparity between the global median age of diagnosis and that observed for both sexes in Ho Chi Minh City.

In contrast, western countries have seen an upward trend in the mean age of HNC diagnosis over the past several decades [223]. The U.S., for example, documented an increase mean age at diagnosis for all HNC subtypes from 1975 to 2016, with the sole exception of oropharyngeal cancer [224]. This subtype saw a decrease in mean age of diagnosis, paralleled by a surge in proportional prevalence, potentially attributable to a rise in HPV-related oropharyngeal cancers.

In a closer examination of Ho Chi Minh City from 1996 to 2015, the mean age of diagnosis receded over this 20-year span for all HNC subtypes, barring oropharyngeal and nasopharyngeal cancers (refer to Supplementary Table 6, A-C). Oral cancers documented the most significant decline, with the mean age of diagnosis falling from 63 years in 1996-2000 to 57 years in 2011-2015. The average age of diagnosis for nasopharyngeal patients remained relatively static, at 49 in 1996, rising slightly to 50 in 2011 and 2015, an observation consistent with statistics from other Asian countries [225].

A further noteworthy revelation from our research is the higher ASR of overall HNC in men compared to women. This gender discrepancy aligns with prior studies examining HNC trends [226], and points towards a higher prevalence of risk factors such as smoking and alcohol consumption, and HPV infection among men. These behaviours, particularly tobacco and alcohol use, have been recognised as significant risk factors for HNC [227].

In the context of Vietnam, understanding the trends in alcohol consumption and smoking is essential. Over the years, Vietnam has experienced an upward trend in both smoking and alcohol consumption, particularly among men [228]. Evidently, these lifestyle factors could contribute to the observed higher ASR of HNC in men. Efforts to reduce the prevalence of these risk factors could

potentially lead to a decrease in the incidence of HNC and need to be an integral part of any comprehensive cancer control strategy [18]. This highlights the urgency for more aggressive public health measures in this region to mitigate these risk factors, and thus, potentially reduce the incidence of HNC among men.

Differences between our study and existing literature, particularly regarding the prevalence of HPV-related oropharyngeal cancer, could potentially be attributed to a dearth of data on HPV infection rates, especially HPV16, in Vietnam. Intriguingly, our data illustrates a consistent uptick in oropharyngeal cases across the observed years, a trend possibly linked to HPV16. This conjecture warrants a more in-depth exploration, as do the regional factors that may be influencing the observed earlier age of diagnosis and decreasing age trends.

Whilst the escalating incidence of oropharyngeal cancer could signal the significant role of HPV, especially HPV16, as a risk factor, attention needs to be drawn to the potential benefits of an expanded HPV vaccination programme in Vietnam. The demonstrated efficacy of the HPV vaccine in preventing HPV-related cancers is internationally recognised [229]. By 2019, it had been incorporated into the national immunization programs of 100 countries, endorsed by the World Health Organization [230]. Vietnam, where the prevalence of HPV infection varies widely and vaccine coverage remains low, would particularly benefit from this approach.

Simultaneously, the data highlights the imperative for robust HNC detection, given the earlier age of diagnosis and decreasing age trends observed. Delays in cancer diagnosis contribute to heightened risk of premature death and lower survival rates; in late-stage HNC, the overall 5-year survival rate is less than 40% [221]. Hence, aggressive screening strategies for early identification of HNC cases in Vietnam warrant serious consideration.

These conclusions, while significant, should be framed within the strengths and limitations of our study. The data were drawn from a well-established registry, ensuring comprehensive capture of total HNC incidence in Ho Chi Minh City, with data completeness enhanced by the centralised HNC treatment. Nevertheless, our inability to track individual patients for survival analysis and the potential non-generalisability of our findings to rural or non-urban settings, where HNC incidence may be lower, must be acknowledged.

In conclusion, our registry-based study exposes an increasing trend over the past 20 years for all HNC subtypes in Ho Chi Minh City. These findings present a foundation for the development of future cancer control strategies. Given the rapidly expanding population and economy of Vietnam, the implementation of apt health care measures, including HPV vaccination and aggressive cancer screening, is poised to significantly impact future HNC diagnoses and treatments in this region.

Supplementary Tables

Supplementary Table 1. Clinical characteristics of 8,974 people with Head and Neck Cancer in Ho Chi Minh City, 1995-2015. Clinical characteristics that were recorded include grade of cancer and base of diagnosis. “Unknown” refers to missing or not available data.

	Male		Female		Total	
	Cases	(%)	Cases	(%)	Cases	(%)
Number of Cases	6523	73%	2451	27%	8974	
Grade of Cancer						
Grade I	1469	16.37%	636	7.09%	2105	23.46%
Grade II	2088	23.27%	519	5.78%	2607	29.05%
Grade III	912	10.16%	334	3.72%	1246	13.88%
Grade IV	903	10.06%	427	4.76%	1330	14.82%
Unknown	1151	12.83%	532	5.93%	1686	18.79%
Base of Diagnosis						
Clinical only	22	0.25%	14	0.16%	36	0.40%
Clinical tests	472	5.26%	189	2.11%	661	7.37%
Exploratory surgery	104	1.16%	28	0.31%	132	1.47%
Biochemistry /Immunology	1	0.01%	0	0.00%	1	0.01%
Cytology/ Haematology	28	0.31%	4	0.04%	32	0.36%
Histology of metastases	99	1.10%	43	0.48%	142	1.58%
Histology of primary	5788	64.50%	2168	24.16%	7956	88.66%
Unknown	9	0.10%	5	0.06%	14	0.16%

Supplementary Table 2. Clinical characteristics of 8,974 people with Head and Neck Cancer in Ho Chi Minh City, by subtype, 1995-2015. Clinical characteristics that were recorded include grade of cancer and base of diagnosis. “Unknown” refers to missing or not available data.

	Larynx/ Pharynx		Nasopharynx		Oral Cavity		Oropharynx		Total - Head and Neck	
	Case	(%)	Case	(%)	Case	(%)	Case	(%)	Total Cases	(%)
Number of Cases	**1909**	**21%**	**2984**	**33%**	**3029**	**34%**	**1052**	**12%**	**8974**	
Sex										
Male	1756	19.57 %	2071	23.08%	1924	21.44%	772	8.60%	6523	72.69%
Female	153	1.70%	913	10.17%	1105	12.31%	280	3.12%	2451	27.31%
Grade of Cancer										
Grade I	585	6.52%	49	0.55%	1288	14.35%	183	2.04%	2105	23.46%
Grade II	722	8.05%	504	5.62%	960	10.70%	421	4.69%	2607	29.05%
Grade III	195	2.17%	661	7.37%	200	2.23%	190	2.12%	1246	13.88%
Grade IV	3	0.03%	1304	14.53%	7	0.08%	16	0.18%	1330	14.82%
Unknown	404	4.50%	466	5.19%	574	6.40%	242	2.70%	1686	18.79%
Base of Diagnosis										
Clinical only	5	0.06%	23	0.26%	7	0.08%	1	0.01%	36	0.40%
Clinical tests	140	1.56%	257	2.86%	194	2.16%	70	0.78%	661	7.37%

Exploratory surgery	64	0.71%	28	0.31%	31	0.35%	9	0.10%	132	1.47%
Biochemistry /Immunology	0	0.00%	1	0.01%		0.00%		0.00%	1	0.01%
Cytology/ Haematology	11	0.12%	10	0.11%	10	0.11%	1	0.01%	32	0.36%
Histology of metastases	21	0.23%	71	0.79%	34	0.38%	16	0.18%	142	1.58%
Histology of primary	1666	18.56 %	2587	28.83%	2749	30.63%	954	10.63 %	7956	88.66%
Unknown	2	0.02%	7	0.08%	4	0.04%	1	0.01%	14	0.16%

Supplementary Table 3 (A-E). Age distribution of Head and Neck Cancers in Ho Chi Minh City by age, sex and subtype, 1996-2015.

Table 3A: Age distribution of Head and Neck Cancer in Ho Chi Minh City by age and sex, 1996- 2015.

Sex	1996-2000	(%)	2001-2005	(%)	2006-2010	(%)	2011-2015	(%)	Total	(%)
Male										
<30	58	4.71%	58	3.96%	46	2.58%	45	2.21%	207	3.17%
30-39	113	9.18%	128	8.73%	159	8.90%	115	5.64%	515	7.90%
40-49	235	19.09%	306	20.87%	397	22.23%	421	20.64%	1359	20.83%
50-59	229	18.60%	355	24.22%	505	28.28%	722	35.39%	1811	27.76%
60-69	315	25.59%	296	20.19%	335	18.76%	409	20.05%	1355	20.77%
70-79	224	18.20%	252	17.19%	242	13.55%	231	11.32%	949	14.55%
80+	57	4.63%	71	4.84%	102	5.71%	97	4.75%	327	5.01%
All ages	1231		1466		1786		2040		6523	
Female										
<30	40	6.46%	41	6.82%	31	5.03%	35	5.69%	147	6.00%
30-39	74	11.95%	74	12.31%	73	11.85%	75	12.20%	296	12.08%
40-49	88	14.22%	93	15.47%	128	20.78%	118	19.19%	427	17.42%
50-59	76	12.28%	89	14.81%	138	22.40%	157	25.53%	460	18.77%
60-69	118	19.06%	97	16.14%	94	15.26%	95	15.45%	404	16.48%
70-79	156	25.20%	124	20.63%	104	16.88%	74	12.03%	458	18.69%
80+	67	10.82%	83	13.81%	48	7.79%	61	9.92%	259	10.57%
All ages	619		601		616		615		2451	

Supplementary Table 3B: Age distribution of Nasopharyngeal Cancer in Ho Chi Minh City by age and sex, 1996 – 2015.

Sex	1996-2000	(%)	2001-2005	(%)	2006-2010	(%)	2011-2015	(%)	Total	(%)
Male										
<30	43	9.51%	40	8.18%	27	4.82%	33	5.79%	143	6.90%
30-39	72	15.93%	69	14.11%	96	17.14%	62	10.88%	299	14.44%
40-49	112	24.78%	133	27.20%	171	30.54%	170	29.82%	586	28.30%
50-59	91	20.13%	129	26.38%	117	20.89%	180	31.58%	517	24.96%

60-69	97	21.46%	74	15.13%	89	15.89%	73	12.81%	333	16.08%
70-79	34	7.52%	40	8.18%	49	8.75%	38	6.67%	161	7.77%
80+	3	0.66%	4	0.82%	11	1.96%	14	2.46%	32	1.55%
All ages	452		489		560		570		2071	
Female										
<30	25	11.47%	28	12.56%	20	8.40%	19	8.12%	92	10.08%
30-39	45	20.64%	49	21.97%	39	16.39%	42	17.95%	175	19.17%
40-49	58	26.61%	58	26.01%	79	33.19%	60	25.64%	255	27.93%
50-59	43	19.72%	35	15.70%	48	20.17%	54	23.08%	180	19.72%
60-69	25	11.47%	29	13.00%	30	12.61%	31	13.25%	115	12.60%
70-79	21	9.63%	20	8.97%	20	8.40%	25	10.68%	86	9.42%
80+	1	0.46%	4	1.79%	2	0.84%	3	1.28%	10	1.10%
All ages	218		223		238		234		913	

Supplementary Table 3C: Age distribution of Oropharyngeal Cancer in Ho Chi Minh City, by age and sex, 1996 – 2015.

Sex	1996-2000	(%)	2001-2005	(%)	2006-2010	(%)	2011-2015	(%)	Total	(%)
Male										
<30	6	4.80%	10	6.06%	4	1.81%	1	0.38%	21	2.72%
30-39	11	8.80%	21	12.73%	24	10.86%	14	5.36%	70	9.07%
40-49	16	12.80%	30	18.18%	43	19.46%	48	18.39%	137	17.75%
50-59	25	20.00%	34	20.61%	61	27.60%	107	41.00%	227	29.40%
60-69	34	27.20%	28	16.97%	45	20.36%	53	20.31%	160	20.73%
70-79	28	22.40%	30	18.18%	30	13.57%	27	10.34%	115	14.90%
80+	5	4.00%	12	7.27%	14	6.33%	11	4.21%	42	5.44%
All ages	125		165		221		261		772	
Female										
<30	6	10.34%	7	10.94%	4	5.48%	1	1.18%	18	6.43%
30-39	10	17.24%	4	6.25%	12	16.44%	3	3.53%	29	10.36%
40-49	11	18.97%	17	26.56%	11	15.07%	18	21.18%	57	20.36%
50-59	6	10.34%	12	18.75%	28	38.36%	24	28.24%	70	25.00%
60-69	8	13.79%	11	17.19%	10	13.70%	25	29.41%	54	19.29%
70-79	10	17.24%	10	15.63%	5	6.85%	7	8.24%	32	11.43%
80+	7	12.07%	3	4.69%	3	4.11%	7	8.24%	20	7.14%
All ages	58		64		73		85		280	

Supplementary Table 3D: Age distribution of Pharyngeal and Laryngeal Cancer in Ho Chi Minh City, by age and sex, 1996- 2015.

Sex	1996-2000	(%)	2001-2005	(%)	2006-2010	(%)	2011-2015	(%)	Total	(%)
Male										
<30	1	0.30%	0	0.00%	2	0.44%	1	0.17%	4	0.23%
30-39	2	0.60%	5	1.31%	7	1.55%	6	1.03%	20	1.14%

40-49	46	13.69%	63	16.45%	60	13.25%	78	13.36%	247	14.07%
50-59	62	18.45%	77	20.10%	156	34.44%	222	38.01%	517	29.44%
60-69	112	33.33%	102	26.63%	93	20.53%	143	24.49%	450	25.63%
70-79	86	25.60%	108	28.20%	95	20.97%	100	17.12%	389	22.15%
80+	27	8.04%	28	7.31%	40	8.83%	34	5.82%	129	7.35%
All ages	336		383		453		584		1756	
Female										
<30	2	4.55%	0	0.00%	0	0.00%	1	3.57%	3	1.96%
30-39	1	2.27%	4	10.81%	5	11.36%	0	0.00%	10	6.54%
40-49	5	11.36%	1	2.70%	8	18.18%	1	3.57%	15	9.80%
50-59	4	9.09%	8	21.62%	6	13.64%	5	17.86%	23	15.03%
60-69	16	36.36%	8	21.62%	9	20.45%	6	21.43%	39	25.49%
70-79	14	31.82%	12	32.43%	12	27.27%	11	39.29%	49	32.03%
80+	2	4.55%	4	10.81%	4	9.09%	4	14.29%	14	9.15%
All ages	44		37		44		28		153	

Supplementary Table 3E: Age distribution of Oral Cancer in Ho Chi Minh City, by age and sex, 1996 – 2015.

Sex	1996-2000	(%)	2001-2005	(%)	2006-2010	(%)	2011-2015	(%)	Total	(%)
Male										
<30	8	2.52%	8	1.86%	13	2.36%	10	1.60%	39	2.03%
30-39	28	8.81%	33	7.69%	32	5.80%	33	5.28%	126	6.55%
40-49	61	19.18%	80	18.65%	123	22.28%	125	20.00%	389	20.22%
50-59	51	16.04%	115	26.81%	171	30.98%	213	34.08%	550	28.59%
60-69	72	22.64%	92	21.45%	108	19.57%	140	22.40%	412	21.41%
70-79	76	23.90%	74	17.25%	68	12.32%	66	10.56%	284	14.76%
80+	22	6.92%	27	6.29%	37	6.70%	38	6.08%	124	6.44%
All ages	318		429		552		625		1924	
Female										
<30	7	2.34%	6	2.17%	7	2.68%	14	5.22%	34	3.08%
30-39	18	6.02%	17	6.14%	17	6.51%	30	11.19%	82	7.42%
40-49	14	4.68%	17	6.14%	30	11.49%	39	14.55%	100	9.05%
50-59	23	7.69%	34	12.27%	56	21.46%	74	27.61%	187	16.92%
60-69	69	23.08%	49	17.69%	45	17.24%	33	12.31%	196	17.74%
70-79	111	37.12%	82	29.60%	67	25.67%	31	11.57%	291	26.33%
80+	57	19.06%	72	25.99%	39	14.94%	47	17.54%	215	19.46%
All ages	299		277		261		268		1105	

Supplementary Table 4. Comparison of crude and age-standardised rates of Head and Neck Cancers in Ho Chi Minh City to global rates based on data compiled by GLOBOCAN. Data for GLOBOCAN was obtained from the World Health Organisation's (WHO), International Agency for Research on Cancer (IARC) Global Cancer Observatory. GLOBOCAN figures were collected in 2020. Note that this research has analysed data between 1996 and 2015.

Ho Chi Minh 1996-2015 (Per 100,000)	Oral Cavity	Larynx/ Pharynx	Oropharynx	Nasopharynx
Crude rate	2.3	1.5	0.8	2.3
ASR	3.3	2.2	1.1	2.7
World 2020 (Per 100,000)	Oral Cavity	Larynx/ Pharynx	Oropharynx	Nasopharynx
Crude rate	4.8	2.4	1.3	1.7
ASR	4.1	2	1.1	1.5

Supplementary Table 5A. Age standardised rates for Oral cancers in Ho Chi Minh City between 1996 and 2015.

	1996-2000	2001-2005	2006-2010	2011-2015	Overall
Overall					
Crude rate	2.4 (2.2 – 2.5)	2.2 (2.1 – 2.4)	2.2 (2.1 – 2.4)	2.4 (2.2 – 2.5)	2.3 (2.2 – 2.4)
ASR	3.5 (3.3 – 3.8)	3.3 (3.0 – 3.5)	3.1 (2.9 – 3.3)	3.2 (3.0 – 3.5)	3.3 (3.2 - 3.4)
AAPC	-0.7 (-10.3 – 9.9)	-4.9 (-11.5 – 2.1)	3.7 (-4.5 – 12.6)	0.7 (-11.1 – 14.1)	-0.8 (-1.6 – 0.1)
Men					
Crude rate	2.5 (2.2 – 2.8)	2.8 (2.6 – 3.1)	3.2 (2.9 – 3.5)	3.5 (3.2 – 3.7)	3.0 (2.9 – 3.2)
ASR	4.3 (3.8 – 4.8)	4.6 (4.2 – 5.1)	4.9 (4.4 – 5.3)	5.5 (5.0 – 5.9)	4.9 (4.7 – 5.1)
AAPC	4.7 (-5.3 – 15.8)	-3.6 (-6.0 – -1.1)	8.4 (-2.3 – 20.3)	-0.5 (-12.9 – 13.6)	1.7 (0.8 – 2.6)
Women					
Crude rate	2.2 (2.0 – 2.5)	1.7 (1.2 – 1.6)	1.4 (1.2 – 1.6)	1.4 (1.2 – 1.5)	1.6 (1.5 – 1.7)
ASR	2.9 (2.6 – 3.3)	2.2 (2.0 – 2.5)	1.7 (1.5 – 2.0)	1.6 (1.4 – 1.8)	2.1 (1.9 – 2.2)
AAPC	-5.4 (-20.2 – 12.1)	-6.9 (-22.1 – 11.2)	-0.1 (-5.3 – 5.5)	2.5 (-9.2 – 15.9)	-4.0 (-5.1 – -2.8)

Abbreviations: ASR, age-standardised rates; AAPC, average-annual percent change.

Supplementary Table 5B. Age standardised rates for Oropharyngeal cancers in Ho Chi Minh City between 1996 and 2015.

	1996-2000	2001-2005	2006-2010	2011-2015	Overall
Overall					
Crude rate	0.7 (0.6 – 0.8)	0.7 (0.6 – 0.8)	0.8 (0.7 – 0.9)	0.9 (0.8 – 1.0)	0.8 (0.8 – 0.8)
ASR	1.0 (0.8 – 1.1)	1.0 (0.8 – 1.1)	1.1 (0.9 – 1.2)	1.3 (1.1 – 1.4)	1.1 (1.0 – 1.2)
AAPC	14.2 (-12.8 – 49.5)	2.8 (-9.3 – 16.4)	-2.9 (-13.1 – 8.5)	6.7 (1.9 – 11.8)	2.0 (0.5 – 3.4)
Men					
Crude rate	1.0 (0.8 – 1.2)	1.1 (0.9 – 1.3)	1.3 (1.1 -1.5)	1.4 (1.3 – 1.6)	1.2 (1.1 – 1.3)
ASR	1.7 (1.4- 2.0)	1.7 (1.4- 2.0)	2.0 (1.7 – 2.2)	2.2 (1.9 – 2.5)	1.9 (1.8 – 2.1)
AAPC	14.6 (-17.6 – 59.4)	1.4 (-21.5 – 31.0)	-2.9 (-24.1 – 24.2)	5.1 (-3.0 – 13.8)	1.7 (0.0 – 3.4)
Women					
Crude rate	0.4 (0.3 – 0.6)	0.4 (0.3 – 0.5)	0.4 (0.3 – 0.5)	0.4 (0.3 – 0.5)	0.4 (0.4 – 0.5)
ASR	0.5 (0.4 – 0.6)	0.5 (0.4 – 0.6)	0.4 (0.3 -0.6)	0.6 (0.4 – 0.7)	0.5 (0.4 – 0.6)
AAPC	9.6 (-22.0 – 54.0)	4.3 (-17.5 – 32.0)	4.1 (-14.4 – 26.6)	12.0 (-4.9 – 32.0)	1.7 (-0.2 – 3.8)

Abbreviations: ASR, age-standardised rates; AAPC, average-annual percent change.

Supplementary Table 5C. Age standardised rates for Nasopharyngeal cancers in Ho Chi Minh City between 1996 and 2015.

	1996-2000	2001-2005	2006-2010	2011-2015	Overall
Overall					
Crude rate	2.6 (2.4 – 2.8)	2.3 (2.1 – 2.4)	2.2 (2.1 – 2.4)	2.1 (2.0 – 2.3)	2.3 (2.2 – 2.3)
ASR	3.2 (3.0 – 3.5)	2.7 (2.5 – 3.0)	2.6 (2.4 -2.8)	2.6 (2.4 – 2.7)	2.7 (2.6 – 2.8)
AAPC	11.0 (5.5 – 16.8)	-7.9 (-19.7 – 5.6)	5.2 (-16.1 – 32.0)	5.5 (-2.7 – 14.3)	-1.1 (-2.5 – 0.3)
Men					
Crude rate	3.6 (3.3 – 3.9)	3.2 (2.9 – 3.5)	3.2 (3.0 – 3.5)	3.2 (2.9 – 3.4)	3.3 (3.1 – 3.4)
ASR	5.1 (4.6 – 5.6)	4.4 (4.0 – 4.8)	4.2 (3.9 – 4.7)	4.2 (3.8 – 4.5)	4.4 (4.2 -4.6)

AAPC	12.4 (3.3 – 22.4)	-6.1 (-19.9 – 10.1)	9.2 (-13.0 – 37.1)	3.5 (-7.4 – 15.7)	-0.8 (-2.2 – 0.6)
Women					
Crude rate	1.6 (1.4 – 1.8)	1.4 (1.2 – 1.5)	1.3 (1.1 – 1.4)	1.2 (1.0 – 1.4)	1.3 (1.2 – 1.4)
ASR	1.8 (1.6 – 2.1)	1.5 (1.3 – 1.7)	1.4 (1.2 – 1.6)	1.3 (1.1 – 1.5)	1.5 (1.4 – 1.6)
AAPC	6.4 (-3.7 – 17.4)	-8.1 (-22.2 – 8.5)	1.6 (-23.8 – 35.5)	11.1 (0.2 – 23.1)	-1.5 (-3.1 – 0.1)

Abbreviations: ASR, age-standardised rates; AAPC, average-annual percent change.

Supplementary Table 5D. Age standardised rates for Pharyngeal/ Laryngeal cancers in Ho Chi Minh City between 1996 and 2015.

	1996-2000	2001-2005	2006-2010	2011-2015	Overall
Overall					
Crude rate	1.4 (1.3 – 1.5)	1.3 (1.2 – 1.5)	1.4 (1.3 – 1.5)	1.6 (1.5 – 1.8)	1.5 (1.4 – 1.5)
ASR	2.3 (2.0 – 2.5)	2.0 (1.8 – 2.2)	2.0 (1.8 – 2.2)	2.4 (2.2 – 2.6)	2.2 (2.1 – 2.3)
AAPC	5.6 (-13.6 – 29.0)	-6.9 (-19.4 – 7.7)	3.8 (-7.6 – 16.6)	9.2 (-6.9 – 28.0)	0.7 (-0.6 – 2.2)
Men					
Crude rate	2.7 (2.4 – 3.0)	2.5 (2.3 -2.8)	2.6 (2.4 – 2.9)	3.2 (3.0 – 3.5)	2.8 (2.7 – 2.9)
ASR	5.0 (4.5 – 5.6)	4.6 (4.1 – 5.1)	4.4 (4.0 – 4.9)	5.5 (5.1 – 6.0)	4.9 (4.7 – 5.2)
AAPC	4.2 (-14.3 – 26.7)	-9.1 (-24.8 – 10.0)	0.3 (-11.1 – 13.2)	12.9 (-8.9 – 39.9)	1.0 (-0.7 – 2.7)
Women					
Crude rate	0.3 (0.2 – 0.4)	0.2 (0.2 – 0.3)	0.2 (0.2 – 0.3)	0.1 (0.1 – 0.2)	0.2 (0.2 – 0.3)
ASR	0.4 (0.3 – 0.6)	0.3 (0.2 – 0.4)	0.3 (0.2 – 0.4)	0.2 (0.1 – 0.3)	0.3 (0.2 – 0.3)
AAPC	0.0 (-34.4 – 52.3)	-1.5 (-24.5 – 28.5)	15.8 (-15.8 – 59.2)	6.5 (-14.0 – 31.9)	-3.1 (-5.7 – -0.3)

Abbreviations: ASR, age-standardised rates; AAPC, average-annual percent change.

Supplementary Table 6. Average age of diagnosis by Head and Neck cancer subtype over 5-year periods between 1996 to 2015. A) Males; B) Females; and C) both sexes.

Supplementary Table 6A: Average age of diagnosis by Head and Neck cancer subtype over 5-year periods between 1996 to 2015 - Males

Male	1996-2000	2001-2005	2006-2010	2011-2015	Overall
HNC	56.7	56.2	55.9	56.4	56.3
OPC	58.2	56.1	56.2	57.0	56.8
Oral	58.9	57.8	57.0	57.2	57.6
Larynx	63.6	63.0	61.6	60.6	62.0
Naso	49.6	49.6	50.1	50.8	50.1

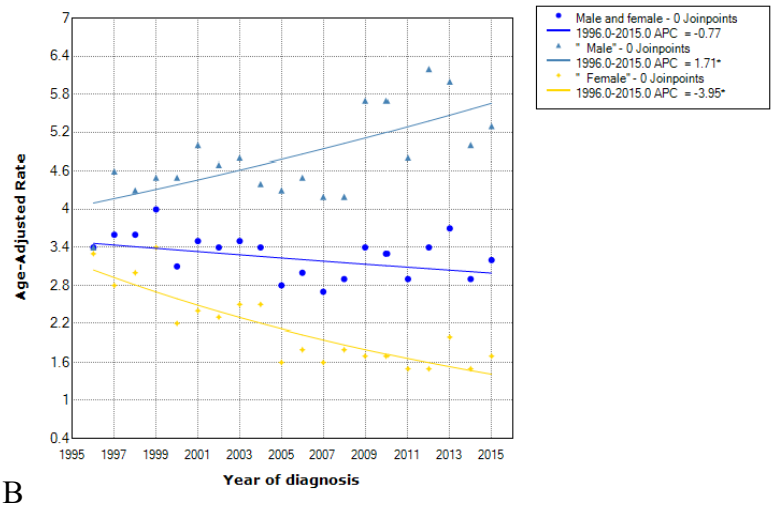
Supplementary Table 6B: Average age of diagnosis by Head and Neck cancer subtype over 5-year periods between 1996 to 2015 - Females

Female	1996-2000	2001-2005	2006-2010	2011-2015	Overall
HNC	58.7	58.1	55.8	55.0	56.9
OPC	53.7	53.2	51.7	58.0	54.4
Oral	67.4	67.2	63.0	57.9	64.0
Larynx	62.4	64.7	60.3	67.4	63.3
Naso	47.2	47.3	48.4	49.3	48.1

Supplementary Table 6C: Average age of diagnosis by Head and Neck cancer subtype over 5-year periods between 1996 to 2015 – Males and Females – Both sexes

Both sexes	1996-2000	2001-2005	2006-2010	2011-2015	Overall
HNC	57.4	56.8	55.9	56.1	56.5
OPC	56.8	55.3	55.1	57.2	56.1
Oral	63.1	61.5	58.9	57.4	59.9
Larynx	63.5	63.2	61.5	60.9	62.1
Naso	48.8	48.9	49.6	50.4	49.5

A



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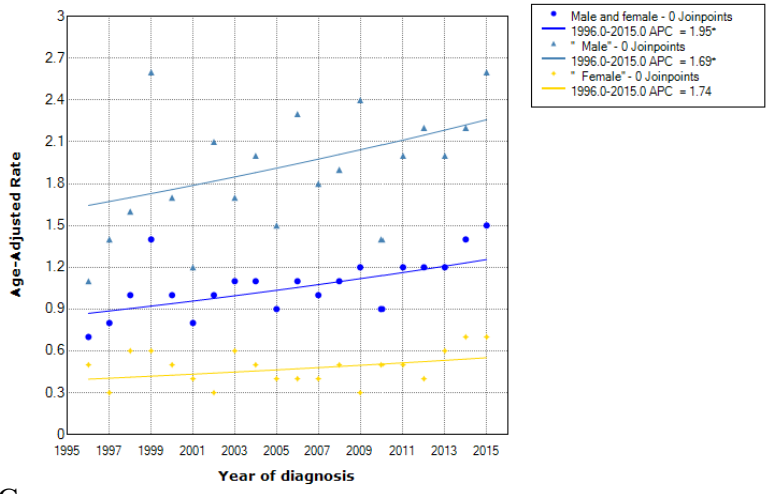

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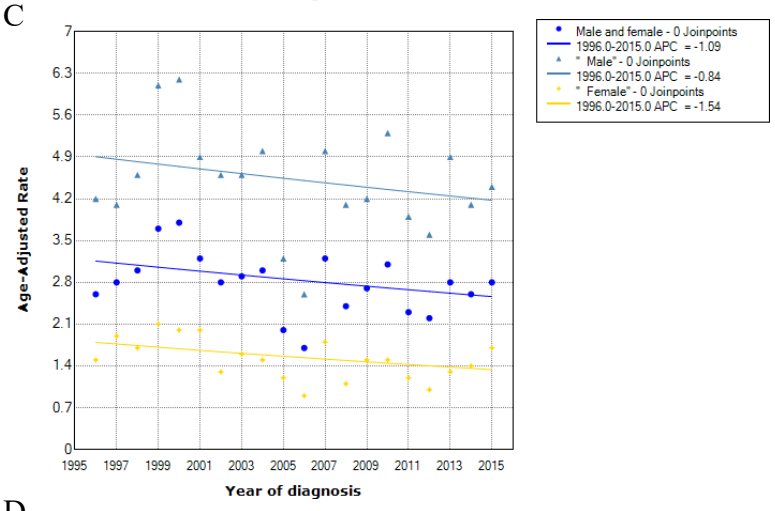

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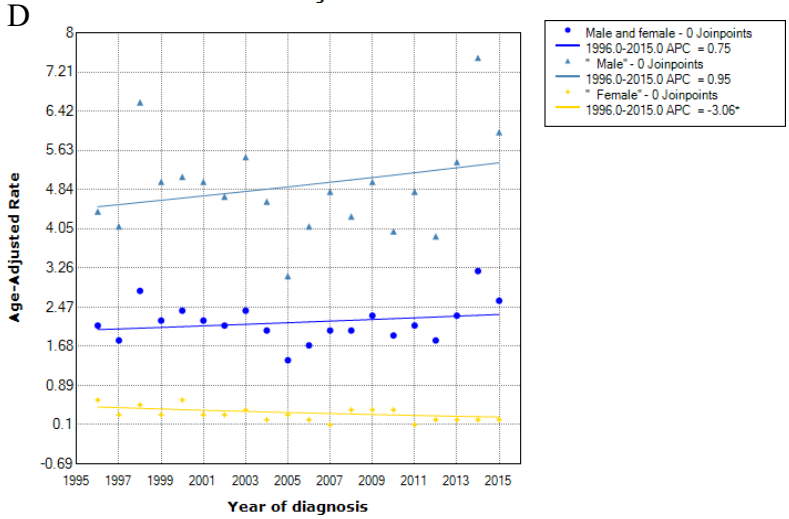

Supplementary Figure 1. Joinpoint regression analysis for A) oral cavity, B) oropharyngeal, C) nasopharyngeal, and D) laryngeal and pharyngeal cancers in Ho Chi Minh City between 1996 and 2015. No joinpoints were identified, as indicated by a lack of change in direction of the “line of best fit” in the age-adjusted rates of the respective head and neck cancers.

Chapter 5

General discussion

5.1 Introduction

Head and neck cancer is recognised globally as the sixth most prevalent form of malignancy. Unfortunately, patients diagnosed in advanced stages face a dismal prognosis with 5-year survival rates falling below 50%. In recent years, there has been an increased association between human papillomavirus (HPV) infections and head and neck cancers. Specifically, HPV has been identified as a leading factor in the progression of oropharyngeal cancers (OPC), a category that includes cancers of the tongue and throat. Notably, a significant majority, approximately 85%, of OPC patients are diagnosed with the high-risk HPV strain, HPV16. Given the mounting evidence that HPV-related OPCs differ in epidemiological, molecular, and clinical profiles from other forms, it's crucial to adapt and refine the current diagnostic and therapeutic strategies. With this backdrop, the primary goal of this doctoral research was to develop a multiplex diagnostic assay for the detection of HPV16 RNA oncogenes E6 and E7 using saliva samples from OPC patients in Sydney, Australia.

However, research often encounters unexpected challenges requiring adaptability. With the onset of the COVID-19 pandemic, a need for a change in research focus emerged. The research then shifted towards bioinformatics and epidemiology to delve into the growth patterns of head and neck cancers in Southeast Asia. Using the Ho Chi Minh Cancer Registry as a resource, this thesis provided a comprehensive analysis of the prevalence and characteristics of head and neck cancers in Vietnam from 1996 to 2015. By focusing on four main subtypes of this malignancy, the research unveiled key trends, offering insights that can inform both clinical practice and public health strategies in the region.

In **Chapter 1**, we present a comprehensive overview of the current understanding and management of HPV-positive OPCs, with a particular focus on the challenges faced by low- and middle-income countries (LMICs). We highlighted that the incidence of HPV-positive OPC is increasing in these resource-limited settings, primarily due to inadequate healthcare infrastructure, limited access to trained healthcare professionals, and a lack of awareness and education about the disease.

Furthermore, we revealed that the delayed diagnosis and treatment of HPV-positive OPC in LMICs result in higher morbidity and mortality rates. This delay can be attributed to various factors, including limited access to healthcare, inadequate healthcare infrastructure, lack of awareness and education about the disease, and the high cost of diagnosis and

treatment options. Moreover, the absence of effective screening programs and the underdiagnosis of the disease further complicate its management in LMICs. This emphasises the need for cost-effective diagnostic tools, such as rapid HPV tests, and the implementation of low-cost treatment options, including chemotherapy and radiation therapy, to address these challenges. Educational programs targeting healthcare professionals and the general public to improve knowledge about HPV-positive OPC, and its prevention is also crucial in curbing the increase in this disease. In addition, we discuss the importance of HPV vaccination as a preventive measure to reduce the burden of HPV-related cancers, including HPV-positive OPC.

The findings underscore the need for a comprehensive and coordinated approach to address the numerous challenges associated with the diagnosis, treatment, and prevention of HPV-positive OPC in these settings. The proposed strategies, including the development of cost-effective diagnostic tools and treatment options, the expansion of educational programs, and the promotion of HPV vaccination, can potentially improve outcomes for patients affected by HPV-positive OPCs in resource-limited settings.

5.2 Advancements in the Diagnosis and Management of HPV-related Oropharyngeal Cancers

Our current understanding of HPV-associated oropharyngeal cancers has evolved significantly in recent years, as researchers continue to uncover the intricate relationship between HPV and the development of these malignancies [231]. OPCs are a subset of head and neck cancers, primarily affecting the tonsils, base of the tongue, and the pharyngeal wall. With an increasing number of cases attributed to HPV infection, the importance of early detection, prevention strategies, and tailored treatment modalities has become a priority in the oncology community [232].

With the incidence of HPV-positive OPCs increasing over the past few decades, there is growing evidence that these tumours have distinct clinical and molecular characteristics compared to their HPV-negative counterparts [231]. The current treatment for OPC patients is multidisciplinary and includes surgery, radiation therapy, and chemotherapy, or a

combination of these treatments. Surgery is the primary treatment for early-stage OPC, while radiation and chemotherapy are used for patients who are not candidates for surgery or have advanced disease. The exact regimen used to treat HPV-positive OPC will depend on various factors including stage and the patient's general health; however, in general, cisplatin is considered the standard chemotherapy drug for the treatment of OPC and is often combined with radiation therapy [233]. The use of targeted therapies, such as cetuximab and other platinum-based agents including carboplatin, has also been shown to improve outcomes in these patients [234, 235].

As discussed in **Chapter 1**, the treatment approach for HPV-positive and HPV-negative OPC is generally individualised based on the patient's specific needs and stage of the disease. Transoral robotic surgery (TORS) is an increasingly common surgical approach that may be used for early-stage tumours and can offer excellent oncological outcomes and better functional outcomes compared to traditional surgery [236]. De-escalation strategies are also being investigated for HPV-positive OPC, which aim to reduce the intensity or duration of treatment to minimize toxicity and improve quality of life without compromising treatment outcomes [232]. In contrast, the HPV-negative OPC treatment approach is often more intensive due to the aggressive nature of the disease. For advanced stages of this disease, in addition to the traditional treatment plan of surgery and combination chemoradiation therapy, high-dose chemotherapy and stem cell transplant may also be considered for select patients.

Several studies have demonstrated that patients with HPV-positive OPC exhibit higher response rates to treatment and better overall survival compared to their HPV-negative counterparts [237, 238]. HPV-positive patients have an approximate five-year survival rate of 70%, whereas HPV-negative patients have a five-year survival rate ranging from 30-50% [237]. Therefore, the accurate identification of HPV status is paramount in determining optimal treatment regimens and predicting patient outcomes.

The failure to identify HPV-positive OPC patients may result in significant consequences for patient outcomes and healthcare costs. This may lead to patients receiving unnecessarily aggressive treatments, which may result in increased morbidity and healthcare costs [232]. Moreover, patients who are not accurately diagnosed with HPV-positive OPC may have lower survival rates compared to those with accurate diagnoses due to a suboptimal and overly toxic treatment approach. Therefore, the identification of HPV status is critical in

developing appropriate treatment plans and reducing healthcare costs while improving patient outcomes.

In light of the strong association between HPV infection and oropharyngeal cancer, prevention strategies have gained significant importance. HPV vaccination has emerged as a powerful tool in reducing the incidence of HPV-associated oropharyngeal cancers [239]. The prophylactic HPV vaccine targets high-risk HPV types, including HPV-16, which is responsible for the majority of HPV-positive oropharyngeal cancers [56]. Widespread vaccination programs have been implemented, targeting both males and females, ideally before their first sexual encounter, to achieve optimal protection [234].

Apart from vaccination, raising public awareness about risk factors for HPV-positive oropharyngeal cancer is crucial in reducing the incidence of this disease. Sexual behaviours, such as having multiple oral sexual partners and a history of sexually transmitted infections, as well as tobacco and alcohol consumption, contribute to the risk of developing HPV-associated oropharyngeal cancer [21]. Public health campaigns aimed at promoting safe sexual practices, reducing tobacco and alcohol use, and encouraging regular dental visits for early detection of oral lesions can help in mitigating the risks.

There is still much to learn about the complex interplay between HPV infection and oropharyngeal cancer development. Ongoing research is focused on understanding the molecular mechanisms underlying the differences between HPV-positive and HPV-negative oropharyngeal cancers, which may inform the development of novel, targeted therapies. Additionally, clinical trials are investigating the efficacy of de-escalation strategies, such as lower doses of radiation therapy or chemotherapy, in HPV-positive patients to reduce treatment-related toxicities and improve the quality of life without sacrificing oncological outcomes [232].

As the understanding of HPV-associated oropharyngeal cancers continues to advance, it is crucial to invest in multidisciplinary research encompassing prevention, early detection, and treatment strategies. This holistic approach will ultimately lead to improved patient outcomes and reduced healthcare costs, alleviating the burden of this disease on patients and healthcare systems.

5.3 De-escalation strategies in HPV-positive OPC planning

De-escalation strategies are an important aspect of the management of OPC patients, with the goal of minimising treatment-related morbidity while maintaining favourable outcomes. The identification of high-risk HPV status has allowed for the development of de-escalation strategies for patients with HPV-positive OPC, as these patients tend to have a more favourable prognosis compared to their HPV-negative counterparts. Therefore, the precise determination of high-risk HPV status is a critical step in the optimisation of treatment approaches for oropharyngeal cancer patients and cannot be overlooked.

One of the most extensively studied de-escalation strategies is the reduction of radiation therapy dose. In a phase II clinical trial (NRG Oncology RTOG 1016), patients with HPV-positive OPC were treated with a reduced radiation dose of 60 Gray (Gy) compared to the standard dose of 70 Gy. The results showed that the reduced radiation dose arm had a similar progression-free survival (hazard ratio (HR) 1.06; 95% confidence interval (CI) 0.79-1.43; p=0.72) and overall survival (HR 1.06; 95% CI 0.78-1.45; p=0.71) compared to the standard dose arm, with fewer acute toxicities (59% vs 78%, p<0.001) and improved quality of life [240]. However, it is important to note that the long-term efficacy of reduced radiation dose is still unknown.

Another de-escalation strategy involves the omission of chemotherapy in selected patients with early-stage HPV-positive OPC. In a phase II clinical trial (ECOG E3311), patients with early-stage disease (T1-2N1) were treated with radiation therapy alone, without chemotherapy. The results showed a two-year progression-free survival rate of 91% and an overall survival rate of 94%, with no difference in toxicity compared to patients who received chemotherapy [241]. This approach may spare patients from the side effects of chemotherapy, such as nausea, vomiting, and neuropathy.

Transoral robotic surgery (TORS) has also emerged as a promising de-escalation strategy for the treatment of HPV-positive OPC. TORS is a minimally invasive surgical technique that allows for the removal of tumours through the mouth without requiring a large incision or external scar. In a retrospective study of 86 patients with HPV-positive OPC

treated with TORS, the three-year progression-free survival rate was 88%, with no difference in survival compared to patients treated with traditional surgical approaches. TORS has also been associated with fewer complications, shorter hospital stays, and improved quality of life compared to traditional surgical approaches [232].

Despite the promising results of these de-escalation strategies, concerns remain regarding the long-term efficacy of these approaches and the potential for disease recurrence. Ongoing clinical trials are exploring various de-escalation strategies to identify the optimal approach to treatment while minimising treatment-related morbidity [242-244]. Accurate testing for high-risk HPV status is an essential component of the treatment planning for patients with oropharyngeal cancer. De-escalation strategies, such as reducing radiation therapy dose, omitting chemotherapy in selected patients with early-stage HPV-positive OPC, and using TORS, have demonstrated promising results in minimising treatment-related side effects while maintaining favourable outcomes.

The exploration of de-escalation strategies in OPC treatment underscores the need for well-structured and extended research. Potential next steps for this field of study could include longer-term clinical investigations, particularly as suggested by the promising research from the NRG Oncology RTOG 1016 trial. In addition, the potential of TORS is evident, however its broader application depends on both technical progression through comparative studies against traditional surgical techniques. In terms of early-stage HPV positive OPCs, further studies could focus on determining specific criteria for omitting chemotherapy, including through the use of biomarkers. It is also essential to look beyond mere clinical outcomes by integrating patient-reported metrics, capturing a more holistic view of patient well-being. Such endeavours will benefit significantly from increased collaboration across medical specialities, allowing a comprehensive approach to OPC case management.

In summary, while the initial data regarding de-escalation strategies for HPV-positive OPC treatment is encouraging, a cautious and methodical approach remains paramount. The objective isn’t only to ascertain the relevance of de-escalation, but to determine how to best refine, apply and oversee these strategies to ensure optimal patient outcomes.

5.4 Addressing Challenges in Diagnosis and Treating HPV-Associated Oropharyngeal Cancer in Low- and Middle-Income Countries

HPV-positive oropharyngeal cancer also constitutes a growing concern in low- and middle-income countries (LMICs) due to the escalating incidence rate and the scarcity of adequate resources for disease management [245]. The global burden of HPV-positive OPC has been steadily increasing, particularly in LMICs, where an estimated 80% of the global cancer burden is expected to occur by 2030 [246]. Diagnosis and treatment for HPV-positive OPC in LMICs often face delays attributable to several factors, including limited access to healthcare, inadequate healthcare infrastructure, insufficient awareness and education, and the prohibitive cost of diagnostic and treatment options [135, 247].

In high-income countries, improved diagnostic tools and screening programs have contributed to a significant decline in the incidence of HPV-negative head and neck cancers [154]. However, the incidence of HPV-positive OPC continues to rise, particularly among men, and is projected to surpass cervical cancer as the leading HPV-related cancer by 2025 [56]. Although the overall 5-year survival rate for HPV-positive OPC is higher compared to HPV-negative OPC, the prognosis remains poor for patients diagnosed at advanced stages [162].

In LMICs, the limited availability of trained healthcare professionals and diagnostic tools presents a major challenge in diagnosing HPV-positive OPC [246]. Patients often must travel long distances to access healthcare facilities, and even when they do, there may be a lack of specialized oncologists or diagnostic equipment, such as imaging systems and pathology laboratories [8]. Moreover, the shortage of trained healthcare professionals' results in a heavy workload for existing personnel, leading to longer waiting times and delayed diagnosis [248]. Additionally, the absence of effective screening programs results in the disease being diagnosed at advanced stages, leading to increased morbidity and mortality rates [8]. Screening programs for HPV-associated cervical cancer, such as the Papanicolaou (Pap) test and the HPV DNA test, have been successful in reducing cervical cancer incidence and mortality in high-income countries, but they are not yet widely available in LMICs due to high costs and insufficient infrastructure [246]. In light of these challenges, emerging technologies like the Viome Discovery™ CancerDetect™ Test for Oral & Throat Cancer offer potential solutions. These non-invasive tests, designed to detect oral and throat cancers using salivary RNA biomarkers,

could significantly simplify the diagnosis process in resource-limited settings. Their adoption might enable more timely and accessible screenings, crucial for early detection and treatment. Furthermore, there is currently no established screening method for detecting HPV-positive OPC at early stages, emphasising the need for developing and implementing cost-effective and accessible screening strategies for this population [247].

A lack of awareness and education regarding HPV-positive OPC risk factors contributes to low screening levels and underdiagnosis [249]. Insufficient knowledge about the disease among healthcare professionals, coupled with cultural and social barriers, hinders the implementation of effective preventive and treatment strategies [246, 249]. In many LMICs, HPV infection and its association with OPC are not well understood by the general population, leading to misconceptions about the disease and a lack of demand for preventive measures [246]. Additionally, social stigma associated with HPV infection, particularly its connection to sexual transmission, may discourage individuals from seeking appropriate care or participating in screening programs [249].

Healthcare professionals in LMICs may also lack adequate training in the detection and management of HPV-positive OPC, further contributing to delays in diagnosis and treatment [248]. This underscores the need for comprehensive educational programs targeted at healthcare professionals to ensure that they are well-equipped to identify and manage HPV-positive OPC cases [8]. Such programs should address not only the clinical aspects of the disease but also the cultural and social barriers that may impede the delivery of effective care [8].

To address these challenges, several initiatives are being undertaken to enhance the diagnosis and treatment of HPV-positive OPC in LMICs. These include developing cost-effective diagnostic tools, such as rapid HPV tests [246], and implementing low-cost treatment options like chemotherapy and radiation therapy [247]. In recent years, research has focused on developing low-cost, point-of-care HPV testing devices that can be easily implemented in resource-limited settings [247]. These devices aim to provide rapid and accurate results, enabling healthcare professionals to make timely diagnoses and initiate appropriate treatment [249].

Vaccination against HPV is another critical preventive measure that can significantly reduce the burden of HPV-related cancers, including HPV-positive OPC [248, 250]. The

World Health Organization (WHO) recommends routine HPV vaccination for girls aged 9-14 years, with catch-up vaccination for older adolescents and young adults [250]. However, the implementation of HPV vaccination programs in LMICs faces several challenges, including limited resources, vaccine supply constraints, and inadequate infrastructure for vaccine delivery [249]. Efforts are needed to expand HPV vaccination coverage in these countries and to raise awareness about the benefits of vaccination in preventing HPV-associated cancers [250].

Diagnosing and treating HPV-positive OPC in LMICs are fraught with challenges related to inadequate healthcare infrastructure, limited resources, and lack of awareness and education. While initiatives aimed at improving access to screening, diagnosis, and treatment are underway, further research and development are required to surmount existing obstacles and enhance outcomes for HPV-positive OPC patients in LMICs. These efforts should encompass the development and implementation of cost-effective diagnostic tools and treatment options, the expansion of educational programs for healthcare professionals and the public, and the promotion of HPV vaccination as a key preventive strategy. Only through a comprehensive and coordinated approach can the growing burden of HPV-positive OPC in LMICs be effectively addressed and mitigated.

5.5 Low-cost Saliva-based Diagnostic Tools for Detection and Monitoring of Head and Neck Cancers

Saliva has emerged as a promising diagnostic medium in recent years, owing to its non-invasive collection method and the ability to detect a diverse range of analytes [251]. The ease and minimal discomfort associated with saliva collection make it an attractive alternative to traditional diagnostic approaches, such as blood draws and tissue biopsies. Salivary testing has also been found to be more applicable to diseases or infections of the oral cavity because saliva is in direct contact with oral tissues, and therefore, it reflects the local physiological and pathological changes occurring in the oral environment [252]. For instance, the proximity of oral diseases, infections and inflammatory processes can result in the release of specific biomarkers into the saliva, making it an ideal biofluid for monitoring oral diseases.

Moreover, saliva collection does not necessitate trained medical personnel, further reducing the cost and complexity of diagnostic testing. Saliva has been shown to detect

various analytes, including hormones, enzymes, proteins, drugs, and infectious agents [252]. Hormones like cortisol and testosterone can be measured in saliva to assess stress levels and evaluate hormonal imbalances. Enzymes, including amylase and lipase, can be measured to evaluate pancreatic function and diagnose pancreatic diseases. Proteins, such as human immunodeficiency virus (HIV) antibodies and antigens, can be identified in saliva to diagnose HIV infection [253]. Additionally, drugs like amphetamines and marijuana can be detected in saliva to assess drug abuse, while infectious agents such as herpes simplex virus (HSV) and cytomegalovirus (CMV) can be detected to diagnose and monitor infections [254, 255].

Despite saliva's potential as a diagnostic fluid, only a limited number of FDA-approved saliva-based liquid biopsy tests are currently available. One such test is the OraQuick Rapid HIV-1/2 Antibody Test, which employs oral fluid collected from the gum line to detect HIV-1/2 antibodies [256]. Another test, the OralCDx BrushTest, is utilised for oral cancer screening by collecting cells from the oral cavity surface. However, this test does not qualify as a liquid biopsy test, as it does not use saliva as the diagnostic fluid [257]. Future research and development efforts in the field of salivary diagnostics hold the potential to expand the availability of saliva-based liquid biopsy tests, further emphasising the value of saliva as a diagnostic medium.

In **Chapter 2**, we conducted a systematic review of PubMed to identify studies characterising the non-coding RNAs present in saliva from patients with oral squamous cell carcinoma. Our review provides a comprehensive summary of the current state of salivary miRNAs and their potential applications for oral cancer diagnosis. However, the limited number of studies in the field of salivary biomarkers and the varied methodologies adopted for processing and measuring salivary miRNAs make it difficult to compare studies and determine the accuracy of the reported miRNA biomarkers. The lack of standardisation in saliva collection, storage, and downstream processing significantly affects the reproducibility of salivary biomarker results.

Our review discussed the challenges and limitations associated with the use of salivary miRNAs as diagnostic biomarkers for oral cancers. One key challenge is the validation and standardisation of salivary miRNA biomarkers. The studies included in the review were often limited by small sample sizes, and the reported miRNAs were not always

consistent across different studies. To validate the diagnostic potential of these miRNAs and establish a consensus on the most relevant biomarkers for oral cancer detection, larger, well-designed studies with diverse populations are needed.

Another critical issue is the standardisation of sample collection, processing, and storage methods, which is crucial to ensure the reproducibility and reliability of salivary miRNA studies. Differences in these methods can affect miRNA detection and quantification, making comparisons between studies challenging.

The development of appropriate detection and quantification methods is also essential for the successful translation of salivary miRNAs into clinical practice. Currently, quantitative real-time polymerase chain reaction (qRT-PCR) is the most commonly used technique for miRNA analysis [258]. However, it has limitations, including the need for a proper endogenous control and the potential for amplification bias [258]. Novel techniques, such as next-generation sequencing (NGS) and digital PCR, show promise in overcoming some of the limitations of qRT-PCR [259]. However, these methods may be expensive and require technical expertise, posing challenges for their widespread adoption [259].

While salivary miRNA biomarkers show promise in oral cancer diagnosis, it is unlikely that they will replace traditional diagnostic methods entirely. Instead, they may serve as a complementary tool, contributing additional information for more accurate and comprehensive monitoring and diagnosis of disease. The development of effective diagnostic algorithms that integrate salivary miRNA biomarkers with clinical examination, imaging, and other molecular markers could improve the overall accuracy and efficiency of oral cancer detection.

Optimising saliva collection and RNA isolation methodologies offer several benefits, including improved diagnostic accuracy. Numerous studies have demonstrated that optimal saliva collection methods and RNA isolation protocols can increase the sensitivity and specificity of RNA-based assays. For example, Gandhi et al. (2020) showed that optimising the RNA isolation protocol increased the yield of RNA and improved the sensitivity of the assay for detecting oral cancer biomarkers [260]. Similarly, Sherrill-Mix et al. (2021) found that using an optimised RNA isolation protocol using reverse transcription and loop-mediated

isothermal amplification (RT-LAMP) which improved the sensitivity of the assay for detecting SARS-CoV-2 RNA in saliva samples [261].

Another benefit of optimising saliva collection and RNA isolation methodologies is increased sensitivity. Several studies have shown that RNA isolated from saliva can detect various diseases, including cancer, infectious diseases, and genetic disorders [262-265]. However, the sensitivity of RNA-based assays can be affected by the quality and quantity of RNA obtained from saliva samples. Therefore, optimising saliva collection methods and RNA isolation protocols can increase the sensitivity of downstream assays.

Reduced sample-to-sample variability is another benefit of optimising saliva collection and RNA isolation methodologies. Variability in RNA quality and quantity can affect the reproducibility and reliability of downstream assays. Optimising saliva collection methods and RNA isolation protocols can help reduce sample-to-sample variability, thereby improving the reproducibility and reliability of downstream assays. Pandit et al (2013) demonstrated that optimising the RNA isolation protocol improved RNA yield the reproducibility of downstream gene expression assays [266].

It is evident that standardising saliva biomarker methodology is paramount for this field of research. To this end, **in Chapter 2** we proposed a guide for the collection and processing of miRNAs in saliva. The adoption of a universal and common methodology would reduce the variations found in current studies and bring us closer to discovering potential salivary miRNAs for use as clinical tools for oral cancer diagnosis.

In summary, we recommend collecting 3-5mLs of saliva from participants, as available. After collection, the sample should be snap-frozen on dry ice and stored at temperatures between -20°C to -80°C for later processing. Our laboratory adopted a liquid-based guanidine isothiocyanate (TRI-Reagent-LS) method for isolating total RNA from saliva. We isolated total RNA in 400µL batches and combined the suspended RNA to augment the quantity. Furthermore, the RNA underwent an overnight incubation in isopropanol, and the RNA pellet was thoroughly washed in 70% ethanol.

The development of a saliva test to detect diseases and infections, particularly human papillomavirus (HPV) and oral cancers, would represent a breakthrough in technology and clinical point-of-care diagnostics. Currently, the gold standard for diagnosing HPV infection is polymerase chain reaction (PCR) testing on cervical swabs for women, while oral cancer diagnosis relies on tissue biopsy and histopathological examination. Both methods are invasive and costly for patients. A saliva-based diagnostic test could revolutionise the detection of HPV and oral cancers by providing a non-invasive, rapid, and cost-effective alternative to current diagnostic methods [267].

Moreover, the development of saliva-based point-of-care diagnostic devices, such as biosensors or lab-on-a-chip platforms, could enable rapid, real-time detection of oral diseases at the patient's bedside or in remote settings [268]. This would not only facilitate early diagnosis and treatment but also enable better patient monitoring and follow-up, ultimately improving patient outcomes and reducing healthcare costs.

The future of salivary testing for monitoring and diagnosing diseases appears to be promising, particularly concerning diseases or infections of the oral and pharyngeal cavities. As a diagnostic tool, salivary testing offers several advantages over traditional diagnostic methods, such as non-invasiveness, ease of collection, and cost-effectiveness [251]. Advancements in salivary diagnostics research could lead to the development of novel saliva-based liquid biopsy tests, improving early detection and management of various diseases and conditions. However, further research, standardisation, and validation efforts are needed to fully realise the potential of saliva as a diagnostic medium and to expand the availability of FDA-approved saliva-based diagnostic tests.

Our lab recognised the growing potential of salivary diagnostics and took the initiative to gather leading experts in the field. With the goal of forming an international salivary diagnostics consortium, we held holding monthly meetings to discuss and coordinate our efforts. The response has been positive, with participants keen to establish consistent protocols and standards, akin to the MIQE guidelines in PCR. The pandemic introduced some delays in our progress, but our team remains committed and prepared to continue our important work in the field.

5.5. A Salivary Liquid Biopsy for Non-Invasive HPV16 Detection in Oropharyngeal Cancer Patients

In **Chapter 3**, we developed and optimised a non-invasive method for detecting transcriptionally active high-risk HPV16 in oropharyngeal cancer (OPC) patients using multiplex qRT-PCR analysis of saliva samples. The key findings of this research contribute to our understanding of HPV16 detection and have potential implications for future screening and surveillance programs.

HPV16 oncogenes E6 and E7 play a key role in the development of HPV-associated cancer by disrupting the normal cell cycle and promoting uncontrolled cell growth [269]. In addition to their role in disrupting the normal cell cycle, E6 and E7 may also play a role in the immune evasion of HPV-positive cancer cells. E6 has been shown to interfere with the function of the immune system by inhibiting the production of interferon, a protein that plays a key role in the immune response to viral infections [270]. E7 has been shown to inhibit the activity of natural killer cells, a type of immune cell that plays a key role in the immune response to cancer [270]. When E6 and E7 are expressed at high levels in HPV-positive cancer cells, they function by targeting and inactivating two key tumour suppressor proteins, p53 and pRb, respectively [271]. P53 is a protein that helps to regulate the cell cycle and promote cell death in response to DNA damage. E6 targets p53 for degradation, allowing cells with damaged DNA to continue dividing and increasing the risk of cancer [271]. PRb is a protein that helps to regulate the cell cycle and inhibit cell growth. E7 inactivates pRb, allowing cells to continue dividing and increasing the risk of cancer [271].

E6 and E7 are particularly useful biomarkers for the detection of HPV because they are expressed at high levels in HPV-positive cancer cells but are not typically expressed in HPV-negative cancer cells or normal cells [154]. This makes them specific markers for HPV infection and allows for the identification of HPV-positive cancer cells even in the absence of detectable virus particles.

Our study successfully detected E6 and E7 mRNA transcripts in HPV16 positive cell lines, SiHa and Caski, with as low as 100pg of input RNA. This demonstrates the sensitivity of the multiplex qRT-PCR method in detecting transcriptionally active HPV16 in these cell

lines. Furthermore, transcriptionally active E6 and E7 mRNA were found exclusively in p16 positive patient tissues, and increasing levels of mRNA expression were observed in conjunction with increased RNA input. This finding highlights the specificity of the assays for detecting HPV16 in patient tissue.

We also explored various saliva collections methods:

1. Collection of whole saliva with no additives: This method involves collecting whole, unprocessed saliva directly from the patient without using any additives. The saliva is then used for downstream gene expression testing.

2. Collection of saliva using a commercial kit (containing preserving additives): In this method, a commercial saliva collection kit was used. These kits usually contain additives that help preserve the quality and integrity of the saliva sample, allowing for more accurate and reliable testing.

After collecting saliva using both methods, total RNA was isolated following our published methodology (refer to **Chapter 2**). We found that the mean total RNA concentration for the non-commercial kit was 67ng, which was significantly lower in yield compared to the mean total RNA concentration for the salivary supernatant collected using the commercial kit at 1,775 ng. Although the total RNA concentrations still showed some variation in both methods, the commercial kit performed better overall in terms of RNA yield and provided usable material for PCR amplification across various gene targets.

Although improvements to RNA yield were made using a commercial salivary collection kit, we also considered the application of RNA isolation columns to improve RNA purity. Whilst there have been several studies that compared the Tri-Reagent liquid RNA isolation method with RNA isolation columns for saliva samples, the results have been inconsistent. Pandit et al (2013) found that an in-house QIAzol lysis reagent produced a 10-fold increase in yield of total RNA from saliva compared to a commercial isolation kit. More recently, Gandhi et al (2020) also found that a modified TRIzol method for RNA extraction from whole saliva provided a higher RNA yield compared to a commercial spin column-based method [260]. Despite this, there are many successful studies that continue to use column-based methods to extract RNA from saliva [81, 86, 272]. While commercial kits have

several benefits, including sample preservation, standardisation and ease of use, these kits can be more expensive than non-commercial kits. Optimisation is vital to whichever method is used to ensure accuracy and reliability, which are critical for the success of the experiment or diagnostic test.

Our research also confirmed the presence of E6 and E7 mRNA in the saliva samples of p16 positive HNC patients, further establishing the specificity of the assays. Among the two assays, the E6 assay demonstrated slightly better performance than the E7 assay, with a mean Cq value of 31.5 compared to 34.4, respectively. This observation suggests that the E6 assay might be a more reliable option for detecting HPV16 in saliva samples, however the ability to multiplex both E6 and E7 adds to the assay's sensitivity.

The use of saliva testing offers several advantages over blood and tissue-based testing methods. Most notably, it is a non-invasive and convenient approach for sample collection, making it a promising option for widespread screening or HPV16 surveillance programs. This could potentially lead to improved patient outcomes through early detection and monitoring of HPV16-associated OPC.

Despite these promising results, we acknowledged that the qPCR method only detects RNA, which may lead to the possibility of false negatives. To address this limitation, further development of the assay to include other HPV16 targets is recommended. Moreover, to fully assess the sensitivity and specificity of this salivary qPCR method, future research should involve a larger cohort of patients.

The development and optimisation of a non-invasive method for detecting transcriptionally active high-risk HPV16 in OPC patients using multiplex qRT-PCR analysis of saliva samples show promising results. The advantages of this method could potentially make it a viable option for widespread screening and HPV16 surveillance programs, ultimately improving patient outcomes through early detection and monitoring.

In practice, we envision this salivary liquid biopsy fitting seamlessly into the current landscape of diagnostics, particularly as a companion test. Given its non-invasive nature and the specific detection of transcriptionally active high-risk HPV16, it could serve as a frontline diagnostic tool for initial screening, especially in large-scale programs. Following an initial

screening with this test, other diagnostic methods could then be applied for confirmation or further investigation. In addition, regular screenings could benefit patients who are at a higher risk or are already diagnosed and need frequent monitoring. Our assay's ability to detect the presence of E6 and E7 mRNA in saliva samples, with an increased sensitivity when both are multiplexed, further underscores its potential as an efficient screening tool. Its comparative advantage over blood and tissue-based tests, in terms of non-invasiveness and convenience, makes it a very promising option.

5.6 Epidemiology of Head and Neck Cancer in Southeast Asia

Owing to the COVID-19 restrictions, the doctoral research project shifted focus to bioinformatics and epidemiology, examining the increasing prevalence of head and neck cancers in Southeast Asia. By leveraging the Ho Chi Minh Cancer Registry, we characterised head and neck cancer cases and their four primary subtypes—oral cancer, oropharyngeal cancer, nasopharyngeal cancer, and laryngeal cancer—in Vietnam from 1996 to 2015. Utilising a bioinformatics approach, this study aimed to quantify the incidence of head and neck cancer in Ho Chi Minh City, analyse age-standardised rates of head and neck cancer, and investigate points of incidence through joinpoint regression analysis.

The landscape of head and neck cancers in Southeast Asia is intricate, with variations among countries. Head and neck cancers rank among the most common cancer type in the region [191]. The incidence of these cancers is typically higher in men than women and increases with age [273]. Tobacco and alcohol consumption are significant risk factors for head and neck cancers in Southeast Asia, as in other regions worldwide. Furthermore, certain head and neck cancers, such as oropharyngeal cancer, have been associated with human papillomavirus (HPV) infection [191].

The prevalence of head and neck cancer subtypes varies by country in Southeast Asia. Vietnam, like Taiwan and the Philippines, faces a notable health challenge from oral cancers. Taiwan registers over 5,000 new cases annually [212], and in the Philippines, the incidence is 2 for every 100,000 people [213]. In China and Hong Kong, nasopharyngeal cancer stands out as the predominant HNC subtype, and it represents a significant number of cases in Vietnam too [214, 215]. Contrarily, in Western nations, there has been a marked increase in

oropharyngeal cases due to the rise in HPV infections [216]. However, as our research demonstrates, in Vietnam, this subtype represents a smaller part of HNC cases.

Analysing lifestyle risk factors, a significant correlation has been observed between tobacco and alcohol use and the incidence of head and neck cancers across Southeast Asia [191].For instance, Indonesia reports a high prevalence of tobacco and alcohol use, with a correspondingly high incidence of head and neck cancers at 14.2 per 100,000 population [248, 273-275]. Similarly, Vietnam, the Philippines, and China present a high incidence of head and neck cancers, which can be attributed to the substantial prevalence of tobacco and alcohol use [213, 214, 275, 276]. Conversely, Thailand and Malaysia, which report lower rates of tobacco use, still exhibit a relatively high incidence of these cancers, suggesting that other factors are at play [200, 227].

Mirroring global trends, HPV infection has also emerged as a significant risk factor for the incidence of oropharyngeal cancers in Southeast Asia. Recently, Argirion et al. (2020) showed that Thailand is experiencing an etiological transition toward HPV-positive OPC, mirroring trends observed in Western nations. By 2030, the prevalence of HPV-positive OPC is projected to surpass 50% in Thailand [277]. Additionally, certain head and neck cancers, such as nasopharyngeal cancer, have been associated with EBV infections [278]. The prevalence of HPV and EBV infections and the corresponding incidence of associated head and neck cancers demonstrate significant variations across the Southeast Asian countries and may be attributed to a combination of genetic, environmental factors, and cultural practices [278]. Despite this, the overall incidence of head and neck cancers in the region remains relatively low compared to other types of cancer [279].

5.7 Trends in head and neck cancer incidence in Ho Chi Minh City between 1996-2015

Our analysis presented in **Chapter 4**, focusing on Ho Chi Minh City in Vietnam, found that head and neck cancers are steadily growing in this area. The study presented a comprehensive analysis of Head and Neck Cancer cases over two decades in Ho Chi Minh City, Vietnam. A total of 8,974 HNC cases were diagnosed, with males constituting 73% of these cases. The investigation focused on four subtypes of HNC, including oral, nasopharyngeal,

oropharyngeal, and laryngeal/pharyngeal cancers. The aim of this research was to quantitate the incidence of head and neck cancer in Ho Chi Minh City, Vietnam, analyse age-standardised rates of head and neck cancer and examine defining points of incidence through joint point regression analysis. Of particular note, our research found that the typical age of diagnosis in Vietnam was around 10 years earlier than global averages, and the mean age of diagnosis decreased over the 20-year study period, which contrasts with trends in Western countries.

Among Vietnamese HNC cases, oral and nasopharyngeal cancers were predominant, in line with trends in Taiwan, Philippines, China, and Hong Kong [191]. The age-standardised rate (ASR) for nasopharyngeal cancers, were also nearly double that of global data.

Oropharyngeal cancers, which have increased in Western countries due to HPV infections, accounted for a smaller portion of HNC cases in Vietnam. However, the study observed a gradual increase in oropharyngeal cancers over 20 years, potentially attributable to HPV type-16. With the coverage of the HPV vaccine in Vietnam traditionally low, our findings imply that a more aggressive screening strategy for identifying cancer cases earlier is warranted, and the expansion of the coverage of the national vaccination target to males would be beneficial given the steady increase in oropharyngeal prevalence in Ho Chi Minh City patients.

Interesting disparities were found in the median age of diagnosis for various HNC subtypes in Vietnam, with a notable 10-year disparity between the global median age of diagnosis and the local median age. Significant disparities were identified in the median age of diagnosis for various HNC subtypes. For laryngeal and oral cancers, the median age of diagnosis was higher in females (67 years for both subtypes) than in males. Conversely, for oropharyngeal and nasopharyngeal cancers, males presented at an older median age than females. The median age of diagnosis for males was 56, a year younger than the median for females at 57. This observed median age of diagnosis in Ho Chi Minh City is a decade younger than the global median age of diagnosis for non-virally associated Head and Neck Squamous Cell Carcinoma (HNSCC), underscoring a striking regional difference.

Another notable finding in the study was the decreasing mean age of HNC diagnosis over the two-decade study period for all subtypes except for oropharyngeal and nasopharyngeal cancers. This contrasts with Western countries, which have seen an upward trend in the mean age of HNC diagnosis.

Our study also revealed a higher age-standardised rate (ASR) of overall HNC in men compared to women, possibly linked to a higher prevalence of risk factors such as smoking, alcohol consumption, and HPV infection among men. With Vietnam's upward trend in both smoking and alcohol consumption, these risk factors may be contributing to the higher ASR of HNC in men, necessitating urgent public health measures to mitigate these factors.

The potential influence of HPV, particularly HPV16, in the increasing incidence of oropharyngeal cancer underscores the need for further research and potential implementation of an expanded HPV vaccination program in Vietnam. Given the observed earlier age of diagnosis and decreasing age trends, the study also highlights the need for more aggressive HNC screening strategies.

The study's findings are based on a well-established registry, providing a comprehensive picture of HNC incidence in Ho Chi Minh City. However, limitations include the inability to track individual patients for survival analysis and the potential non-generalisability of the findings to rural settings.

In summary, this study unveils an increasing trend over the past 20 years for all HNC subtypes in Ho Chi Minh City and provides a crucial foundation for developing future cancer control strategies. Addressing the major risk factors, such as tobacco and alcohol use, as well as increasing HPV vaccination coverage, can significantly impact the overall burden of head and neck cancers in the region. Furthermore, continued research into the genetic and environmental factors contributing to head and neck cancers in Southeast Asia will help to inform targeted prevention and treatment strategies.

5.8 Conclusion

This doctoral research project aimed to provide a deeper understanding of the role of the human papillomavirus and its oncogenes in head and neck cancers, to develop a non-invasive method for detecting HPV, and to conduct an epidemiological study of HNCs in Southeast Asia, particularly in Vietnam. This research supported the value of non-invasive diagnostic tools and highlighted saliva as a promising diagnostic medium.

We started with a systematic review, to identified and summarised studies exploring non-coding RNAs in the saliva of patients with oral squamous cell carcinoma. While these studies show promise, several challenges such as the validation and standardisation of salivary miRNA biomarkers, as well as sample collection, processing, and storage methods, were identified. These findings highlight the need for ongoing research and development in the field of salivary diagnostics.

Furthermore, the research underscored the need to optimise saliva collection and RNA isolation methodologies to improve diagnostic accuracy, increase sensitivity, and reduce sample-to-sample variability. This optimisation could provide several benefits, including the potential to increase the sensitivity and specificity of RNA-based assays, enabling more accurate detection of diseases such as HPV and oral cancers.

Following this, the doctoral research shifted to a novel non-invasive method for detecting transcriptionally active HPV16 using multiplex qRT-PCR analysis of saliva samples from OPC patients. The goal was to identify E6/E7 mRNA of HPV16, indicating the presence of the transcriptionally active virus. Preliminary results demonstrated the potential of this salivary qPCR method for early detection and monitoring of HPV16 in OPC patients. The results highlighted the possibility of revolutionising the way HPV16-associated OPC is diagnosed and monitored, improving patient outcomes through more widespread screening programs. However, the method's sensitivity and specificity require further assessment via research involving larger patient cohorts.

Lastly, we conducted an epidemiological study of HNCs in Southeast Asia, focusing on Ho Chi Minh City, Vietnam. Using data from the Ho Chi Minh Cancer Registry, the study

explored the incidence, trends, and subtype distribution of HNCs in this region over two decades (1996 to 2015). The findings showed an increase in HNC subtypes, with males representing 73% of cases. An intriguing trend was the decrease in the typical age of diagnosis for HNCs in Vietnam, contradicting global trends and emphasizing the need for more aggressive HNC screening strategies.

The research also found a gradual increase in oropharyngeal cancers, potentially linked to HPV16, paralleling global trends. This suggested the need for an expanded HPV vaccination program in Vietnam, specifically targeting males. The research additionally pointed out lifestyle risk factors for HNCs, such as tobacco and alcohol use, warranting public health interventions.

This research undertaken has advanced our understanding of HPV's and its association with HNCs. We have optimised a novel approach for the detection of HPV16, using saliva. This newly proposed method may hold the promise of reshaping the landscape of HNC diagnostics, making it more efficient and accessible. The epidemiological findings derived from Vietnam reinforce the urgency for more intensive screening methodologies, broader application of HPV vaccinations, and strategically designed public health interventions. This approach will help address the considerable health impact of HNCs more efficiently and sustainably.

Building on the insights gained from the studies presented in this thesis, future research should aim to further refine and expand the application of saliva-based diagnostic assays. As our approach has shown promising results for early detection of HPV16 in oropharyngeal cancer patients, future studies should focus on larger, multi-centre trials to validate and standardise these assays, ensuring their reliability and effectiveness across different populations and healthcare settings. This will be crucial for integrating these tools into routine clinical practice, potentially enabling widespread screening programmes that could significantly improve early detection rates of HPV-driven cancers.

Additionally, the initial success of the saliva-based diagnostics highlights an opportunity to explore similar non-invasive methods for other biomarkers associated with HNC. Research could extend to identifying and validating additional salivary biomarkers that predict the presence of other high-risk HPV strains or different cancer subtypes. Concurrently, expanding HPV vaccination programmes and public health campaigns to

educate about HPV's risks and prevention should remain a priority, particularly in regions like Southeast Asia where the burden of disease is high and growing. This holistic approach, combining advanced diagnostics, preventive measures, and robust public health strategies, offers a pathway to significantly reduce the global impact of head and neck cancers.

The exploration of salivary diagnostics, especially for diseases affecting the oral and pharyngeal regions, has also unveiled promising avenues for the future. These innovative diagnostic approaches underscore the immense potential of saliva-based testing in monitoring and detecting various health conditions. As advancements continue to evolve in this field, we foresee a significant transformation in the way diseases are detected and managed, paving the way towards more effective and less invasive healthcare solutions.

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