Clinical impact and limitations of liquid biopsies in HPV-related head and neck cancer: a narrative literature and systematic review

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ABSTRACT – ENGLISH

The incidence of head and neck cancer (HNC) is rising annually. Researchers attribute this increase to the prevalence of human papillomavirus (HPV), the most common sexually transmitted infection worldwide. In fact, the majority of oropharyngeal cancers (OPC) can be attributed to chronic infection with HPV. The current standard of care involves surgery and adjuvant chemoradiation therapy which can often leave survivors debilitated. Unfortunately, long-term side effects of treatment greatly impact the quality of life of HNC patients. In order to limit treatment toxicities and improve patient quality of life, de-escalated treatment strategies are currently being explored. The purpose of this thesis is to understand the translational potential and limitations of liquid biopsies to improve personalized treatment regimens of patients with HPV-related HNC. This topic was explored in a narrative literature review, however, a systematic review (PROSPERO ID: 560498) was also conducted to determine whether the immune microenvironment of HNC patients could be characterized by liquid biopsy. Two bibliographic databases (Medline and Embase) were searched for eligible studies based on MeSH (Medical Subject Headings) terms and keywords. Search terms included cancer, circulating tumor cells, liquid biopsy, and the immune microenvironment. Studies selected from the databases were imported into Covidence software for identification, removal of duplicates, and for screening based on predefined eligibility criteria. 304 studies were identified via the search strategy, and of these, eight studies were retained for data extraction. The studies included in the systematic review were published between 2017 and 2024 and involved 814 HNC patients from three different countries. The results of the literature review suggest that liquid biopsies analyzing circulating tumor DNA (ctDNA) offer potential benefits, such as reduced diagnostic time and biopsy repeatability. Additionally, the applications of liquid biopsies in HNC appear to include diagnostics, disease surveillance, and patient prognostication. Furthermore, the data collected in the systematic review indicates that the immune microenvironment may play a role in predicting responsiveness to treatment in patients with HNC. However, further studies should be conducted to improve and validate these findings within the field.

ABSTRACT – FRENCH

L'incidence du cancer de la tête et du cou augmente chaque année. Les chercheurs attribuent cette augmentation au virus du papillome humain (VPH), la maladie vénérienne le plus répandu dans le monde. En fait, la grande majorité des cancers oropharyngés peuvent être attribués à une infection chronique par le VPH. La norme actuelle de soins comprend la chirurgie et la chimioradiothérapie adjuvante, qui peut souvent laisser les survivants affaiblis. Malheureusement, les effets secondaires à long terme du traitement ont un impact considérable sur la qualité de vie des patients atteints de cancer de la tête et du cou. Afin de limiter les toxicités du traitement et d'améliorer la qualité de vie des patients, des stratégies de traitement moins intenses sont actuellement explorées. Une analyse de la littérature a été réalisée pour évaluer le potentiel translationnel et les limites des biopsies liquides pour améliorer les schémas thérapeutiques personnalisés des patients atteints de ce cancer. En outre, une étude systématique (PROSPERO ID: 560498) a été réalisée pour déterminer si les microenvironnements immunitaires des patients atteints de cancer de la tête et du cou pouvaient être caractérisés par des biopsies liquides. Deux bases de données bibliographiques (Medline et Embase) ont été consultées à la recherche d'études éligibles sur la base de termes MeSH (Medical Subject Headings) et de mots-clés. Les termes de recherche comprenaient le cancer, cellules tumorales circulantes, biopsie liquide, et le microenvironnement immunitaire. Les études sélectionnées ont été importées dans le logiciel Covidence pour l'identification, la suppression des doublons, et la sélection sur la base de critères d'éligibilité prédéfinis. La stratégie de recherche a permis d'identifier 304 études, dont huit ont été retenues pour l'extraction des données. Les études incluses dans la revue systématique ont été publiées entre 2017 et 2024 et ont porté sur 814 patients atteints de cancer dans trois pays différents. Les résultats de l'analyse de la litérature suggèrent que les biopsies liquides analysant l'ADN tumoral offrent des avantages potentiels, tels que la réduction du temps de diagnostic et la répétabilité. En outre, les applications des biopsies liquides dans le cas du cancer de la tête et du cou semblent inclure le diagnostic, la surveillance de la maladie, et le pronostic du patient. De plus, les données recueillies dans le cadre de l'étude systématique indiquent que le microenvironnement immunitaire peut jouer un rôle dans la prédiction de la réponse au traitement chez les patients atteints de cancer cervico-faciale. Cependant, d'autres études devraient être menées pour améliorer et valider ces résultats.

FORMAT OF THE THESIS

This thesis is based on the guidelines provided by the Faculty of Graduate and Postdoctoral Studies of McGill University. It consists of five main chapters: the introduction (Chapter 1), a review of the literature in the field (Chapter 2), a manuscript involving a literature review of the clinical applications and limitations of liquid biopsies in HNC, as well as a systematic review about the potential of using liquid biopsies to characterize the immune microenvironment of HNC patients (Chapter 3), a general discussion (Chapter 4), conclusions and future directions (Chapter 5), a reference list for the citations found outside of the manuscript, and supplementary materials.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Sabrina Wurzba, for providing me abundant guidance and support throughout my graduate studies. Your effort and devotion to your craft does not go unnoticed. I would also like to thank Dr. Bernard Segal for never sugar-coating things when providing me with honest and supremely helpful feedback. In addition, I would like to thank Dr. Mlynarek, Dr. Afilalo, and Dr. Alaoui-Jamali for sitting on my thesis committee and providing me with constructive criticism. Furthermore, I would like to thank Andrea Quaiattini for her immense help with conducting my systematic review.

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CONTRIBUTION OF AUTHORS

Megan Araujo performed the complete writing of the present thesis. Megan Araujo is the first author of Manuscript 1 (Chapter 3). All co-authors of the manuscript, Fatemeh Farshadi, Michael Hier, Marco Mascarella, Alex Mlynarek, Moulay Alaoui-Jamali, and Sabrina Daniela da Silva, contributed to the reviewing of the manuscript. Jenna Bouassaly contributed major revisions to the manuscript. Conception of study design and supervision was done by Sabrina Daniela da Silva. Megan Araujo established the protocol for the systematic review, performed the article screening, data collection, and data synthesis. Fatemeh Farshadi confirmed the data collection and synthesis. Andrea Quaiattini, a senior medical librarian, guaranteed that the proper search strategy and protocol were used in the systematic review. All the images and graphs presented in the paper were created by Megan Araujo using Biorender under the guidance of Sabrina Daniela da Silva. Final approval of the manuscript was done by all the authors involved in the manuscript.

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LIST OF ABBREVIATIONS

ARMS-PCR, amplification refractory mutation system-polymerase chain reaction

BEAMing, polymerase chain reaction involving beads, emulsion, amplification, and magnetics

B7-H3, cluster of differentiation 276

CAR-T, adoptive cell therapy

CASP, critical appraisal skills programme

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CD20: B-lymphocyte antigen

CDK4/6, cyclin-dependent kinase 4 and 6

cfDNA, cell-free deoxyribonucleic acids

CTC, circulating tumor cell

CTC-Chip, circulating tumor cell chromatin immunoprecipitation

ctDNA, circulating tumor deoxyribonucleic acids

CT scan, computed tomography scan

DAB, diaminobenzidine

DC, dendritic cell

ddPCR, digital droplet polymerase chain reaction

DNA, deoxyribonucleic acid

EBRT, extrenal beam radiation therapy

ECM, extracellular matrix

EGFR, epidermal growth factor receptor

Exo-CHIP, chromatin immunoprecipitation with exonuclease treatment

E2F, family of transcription factors

E6AP, E6 associated protein

FDA, food and drug administration

G1, gap phase of the cell cycle

G2, gap 2 phase of the cell cycle

HER2, human epidermal growth factor receptor 2

HNC, head and neck cancer

HNSCC, head and neck squamous cell carcinoma

HPV, human papillomavirus

HPV+, human papillomavirus-positive

HPV-, human papillomavirus-negative

HPV+HNC, human papillomavirus-positive head and neck cancer

HPV+OPC, human papillomavirus-positive oropharyngeal cancer

HRAS, Harvey rat sarcoma virus

HRP-polymer, horseradish peroxidase-polymer

IGRT, image-guided radiation therapy

IHC, immunohistochemistry

ILs, interleukins

IMRT, intensity-modulated radiation therapy

IRF-1, interferon regulatory factor-1

ISH, in situ hybridization

LAG, lymphocyte activation gene 3

LCR, locus control region

M, mitosis

MDM2, mouse double minute homolog 2

MDSC, myeloid-derived suppressor cells

MeSH, medical subject headings

MiRNA, micro ribonucleic acid

MMP, matrix metalloproteinase

MRI, magnetic resonance imaging

NK, natural killer cell

NECTORs, neo-adjuvant chemotherapy followed by transoral surgery and selective neck dissection

NGS, next-generation sequencing

NR, non-responder

NUBP2, nucleotide binding protein 2OPC, oropharyngeal cancer

OPSCC, oropharyngeal squamous cell carcinoma

OS, overall survival

OSCC, oral squamous cell carcinoma

P, phosphate

PCR, polymerase chain reaction

PD-1, programmed cell death protein 1

PD-L1, programmed death-ligand 1

PD-L2, programmed death-ligand 2

PET/CT, positron emission tomography/computed tomography

PIK3CA, phosphatidylinositol-4,5-bisphosphate 3 kinase

PFS, progression-free survival

Post-op, post-operative

Pre-op, pre-operative

PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses

pSTAT3, phosphorylated transducer and activator of transcription 3

P14ARF, alternate reading frame of CDKN2A

R, responder

Rb, retinoblastoma tumor suppressor protein

RFS, recurrence-free survival

RoB, risk of bias

RTK, receptor tyrosine kinase

S, synthesis phase of the cell cycle

SEC, size exclusion chromatography

TAM, tumor-associated macrophage

TGFβ, transforming growth factor-beta

TIL, tumor-infiltrating lymphocyte

TLR4, toll-like receptor 4

TMA, tissue microarray

TME, tumor microenvironment

TNF-α, tumor necrosis factor-alpha

TNM, T: extent of the tumor, N: nodal involvement, and M: metastasis

TORs, transoral robotic surgery

Treg, regulatory T cell

Ub, ubiquitin

VEGF, vascular endothelial growth factor

5-FU, 5-fluorouracil

CHAPTER 1 – INTRODUCTION

1.1 Rationale

Head and neck cancer (HNC) is within the top ten most common cancer types in the world [1]. In the past decade, the prevalence of HNC has been increasing exponentially, even though the most common risk factors, which include smoking and excessive alcohol consumption, have declined [2]. This increase in prevalence can be attributed to infection with human papillomavirus (HPV), a common sexually transmitted disease [3]. When an individual is exposed to chronic HPV infection, they are more susceptible to developing cancer [3]. The current treatment for HNC depends on the clinical stage of the disease and usually involves surgery followed by adjuvant chemoradiation therapy, and immunotherapy for recurrent and metastatic tumors [4]. However, these treatments are aggressive and often leave patients with dysphagia, dysphonia, trismus, ulceration, and hemorrhage [4].

Patients with HPV-related HNC have a relatively favorable prognosis compared to patients with HPV-negative HNC, since their tumors are more susceptible to anti-cancer drugs [5]. Due to this superior prognosis, researchers have postulated whether patients with HPV-related HNC are eligible for a de-escalated course of treatment that would limit toxicities and negative side effects [6]. Recent studies have shown that specialized tumor microenvironments (TME) have the potential to act as cancer therapy targets through reprogramming [7], [8], [9], [10], [11], [12], [13], [14], [15], [16]. However, a major limitation associated with cancer therapy approaches targeting the TME includes primary or acquired resistance due to various extrinsic and intrinsic factors [17]. A deeper understanding of the dynamic TME components and their real-time interaction could help in overcoming these limitations [18]. In this manner, the scientific community aims to integrate minimally invasive liquid biopsy technology into clinical practice, with the goal of improving patient outcomes by monitoring tumor progression during treatment.

A chapter of this thesis explored the potential clinical applications and limitations of liquid biopsies in HPV-related HNC. Furthermore, a systematic review investigated whether the immune microenvironment can be leveraged via liquid biopsy to improve the clinical management of cancer. Therefore, this thesis evaluated the predictive and prognostic value of liquid biopsy as a tool to support HNC diagnosis and treatment.

1.2 Objectives

The primary aim of this thesis is to understand the clinical applications and limitations of liquid biopsies in HPV-related HNC. Furthermore, this study aims to investigate whether the circulating immune factors can be leveraged via liquid biopsy to predict treatment outcomes and prognosis in patients with HNC.

CHAPTER 2 – REVIEW OF THE LITERATURE

2.1 Introduction to Cancer

Cancer is the second most common cause of death in high income countries [19]. Current statistics predict that one in two women and one in three men living in North America will develop cancer in their lifetime [20]. The risk of developing cancer depends on lifestyle factors (such as smoking and alcohol consumption), environmental factors, and socioeconomic status [21]. Cancer is a complex and heterogenous genetic disease which occurs when portions of the genome are mutated or altered [22]. These genetic changes can be inherited (germline mutations) or acquired (somatic mutations) during a person's lifetime due various factors such as environmental exposure (e.g., radiation and carcinogens), lifestyle choices, infection, and random errors in DNA replication [22], [23]. While not all cancers are inherited, the genetic basis of cancer involves mutations in specific genes that regulate cell growth, division, and death [24]. These genes include oncogenes which, when mutated or overexpressed, can promote the growth of cancer cells, tumor suppressor genes which normally prevent uncontrolled cell growth and promote the repair of damaged DNA, and DNA repair genes which are also involved in repairing damaged DNA [24].

Genomic damage can occur through various types of mutations [24]. The key types of mutations that contribute to cancer development include point mutations, chromosomal rearrangements, insertions and/or deletions of nucleotides, and copy number variations [25], [26], [27], [28]. These mutations can accumulate over time due to intrinsic factors like DNA replication errors and extrinsic factors such as exposure to carcinogens, radiation, and certain viruses [22], [23]. The combination of these genetic alterations disrupts normal cellular processes and leads to the development and progression of cancer [22]. Epigenetic reprogramming, such as DNA methylation, acetylation, phosphorylation, ubiquitylation, and SUMOylation, also plays a significant role in cancer development and progression [29], [30]. Changes to the DNA methylome can alter gene expression without permanently modifying the genetic code [31], [32].

Malignancy can also occur when normal cellular regulation mechanisms are defective [33]. Defective cellular regulation can promote de-differentiation, rapid cellular proliferation, evasion of apoptosis and immune surveillance, as well as deregulated metabolism and epigenetics (**Figure 1A**) [7], [33], [34]. These events encompass the hallmarks of cancer and will be explored in a

future section of the present thesis [7]. As a cancer further progresses, the malignant cells can spread into surrounding tissues (**Figure 1B**) [34]. In addition, some cells can intravasate into the circulatory or lymphatic system to form a metastatic colony in a distant organ (**Figure 1B**) [34]. In HNC, distant metastasis most commonly occurs in the lungs (70-85%), the bones (15-39%), and the liver (10-30%), respectively [35], [36]. However, the majority (~70%) of HNC patients experience locoregional metastasis to the cervical lymph nodes [37]. Unfortunately, metastasis is the cause of 90% of all cancer-related deaths [35].

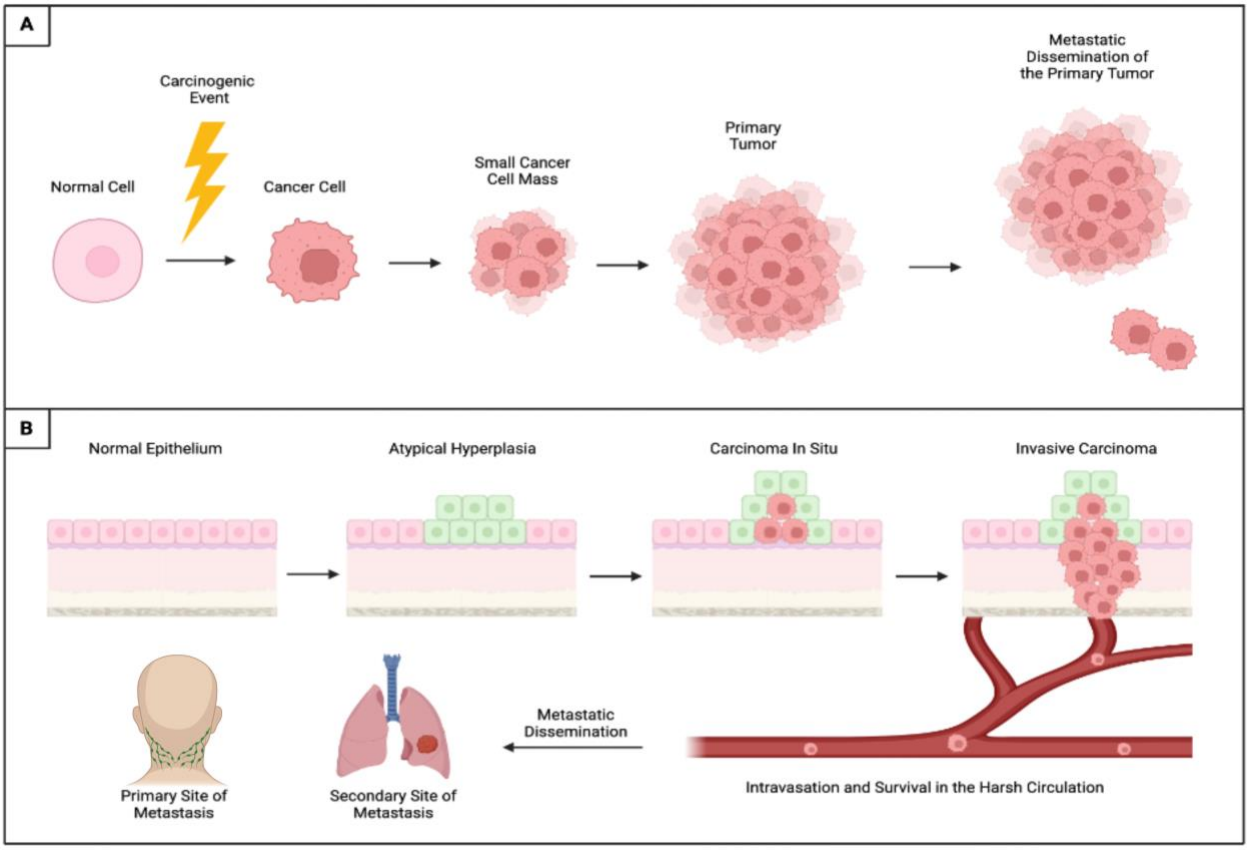

Figure 1. Cancer progression and metastasis. A. Progression of a normal cancer cell to a primary tumor. Primary tumor cells can grow and invade the surrounding tissue, thereby initiating the metastatic dissemination process by shedding tumor cells into the bloodstream or lymphatic system [7], [34]. **B.** Cancer progression in epithelial tissue. This scheme illustrates the stepwise progression from normal epithelium to an invasive carcinoma [34]. In HNC, the main metastatic sites are the cervical lymph nodes and the lungs, respectively [35], [36], [37]. Figure created using Biorender.com. Figure adapted from [38].

Carcinomas can be defined as malignancies that are epithelial in origin, whereas sarcomas can be defined as malignancies that are mesenchymal in origin [39], [40]. Epithelial cells form the lining of the internal organs, body cavities, and the skin [39]. Squamous cells are a subset of

epithelial cells that line the upper aerodigestive tract and the mucosal surfaces of the head and neck region [39]. Mesenchymal cells, on the other hand, are cells that differentiate into connective tissue, lymphatic tissue, and blood vessels [40]. Approximately 85% of all solid tumors are epithelial in origin [41], [42].

At the time of diagnosis, a cancer will be staged based on the clinical and pathological extent of the tumor, the nodal involvement, and if it has metastasized [43]. This staging process, known as TNM staging (tumor (T), node (N), and metastasis (M)), helps classify the severity and the spread of cancer [44]. The lower the cancer stage, the better the prognosis of the patient [43]. Unfortunately, cancer is often diagnosed at later stages of disease progression where tumors are typically larger, more invasive, and often infiltrate surrounding tissues and organs [45]. The characteristics of late stage tumors make them much harder to treat in a curative manner [45].

2.2 The Hallmarks of Cancer

The six hallmarks of cancer, described in 2000 by Hanahan and Weinberg, include sustaining proliferative signaling, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, resisting cell death, and invasion and metastasis [46]. In 2011, Hanahan and Weinberg amended their previous theory by adding emerging hallmarks and enabling characteristics [7]. The emerging hallmarks of cancer include deregulating cellular energetics and avoiding immune destruction, whereas the enabling characteristics include genome instability and tumor-promoting inflammation [7]. These hallmarks collectively contribute to the ability of cancer cells to grow uncontrollably, evade normal regulatory mechanisms, and spread throughout the body [7].

The systematic review conducted in Chapter 3 explores the role of the immune microenvironment in HNC. The immune and inflammatory aspects of HNC are characterized by dynamic interactions between cancer cells, immune cells, stromal cells, and cytokines, which can have profound effects on tumor progression and response to therapy [47]. Multiple studies have demonstrated that an impaired immune system is linked with a high prevalence of several tumor types [48], [49], [50]. For example, anogenital cancers, such as those caused by chronic infection with HPV, are often linked with immune system impairment [51], [52], [53], [54]. Under normal conditions, the immune system is expected to recognize and subsequently eliminate all foreign

antigens present in the bloodstream [7]. However, cancer cells can adapt to overcome immune surveillance mechanisms [7].

One way that malignant cells overcome immune surveillance mechanisms is by acquiring immunosuppressive properties, such as the expression of PD-L1 (programmed death-ligand 1) on their cell surface [55]. In addition, cancer cells may evade the immune system by restricting antigen recognition [56]. Loss of antigenicity can occur when cancer cells lose immunogenic cell surface proteins or when there are defects in antigen processing [55]. Tumors with low antigenicity can evade immune surveillance and consequently proliferate in an uncontrolled manner [55]. Highly immunogenic cancer cell clones, on the other hand, will be rapidly eliminated by the immune system [55].

Chronic inflammation, which is often associated with risk factors such as tobacco and alcohol use, HPV infection, and oral microbiome dysbiosis, contributes to HNC development and progression [57], [58]. A chronically inflamed TME promotes the secretion of chemokines and cytokines into the circulation [7], [59]. These secreted factors can promote tumor cell proliferation, angiogenesis, and invasion, while also inhibiting antitumor immune responses [59]. As malignancies progress from neoplastic tissue to clinically detectable tumors, cancer cells evolve within a highly specialized microenvironment characterized by a corrupted extracellular matrix (ECM) and chronic inflammation, which may activate common signaling pathways that promote tumor growth and metastasis [57].

2.3 Overview of Head and Neck Cancer

HNC is the sixth most common malignancy in the world, accounting for approximately 4% of all cancer diagnoses [60]. HNC involves tumors which originate in the epithelial cells that line the oral cavity, the pharynx, the larynx, the nasal cavity, the paranasal sinuses, and the salivary glands (**Figure 2**) [61]. Although they are located within the head and neck region, malignancies of the brain, thyroid, and eyes do not fall under the umbrella of this cancer type [61]. The most common locations for HNC to occur are in the oral cavity, the pharynx, and the larynx [61]. Due to spatial proximity, HNC tumors commonly metastasize to the cervical lymph nodes [37]. Unfortunately, approximately 70% of HNC patients have nodal metastasis at the time of initial diagnosis [37].

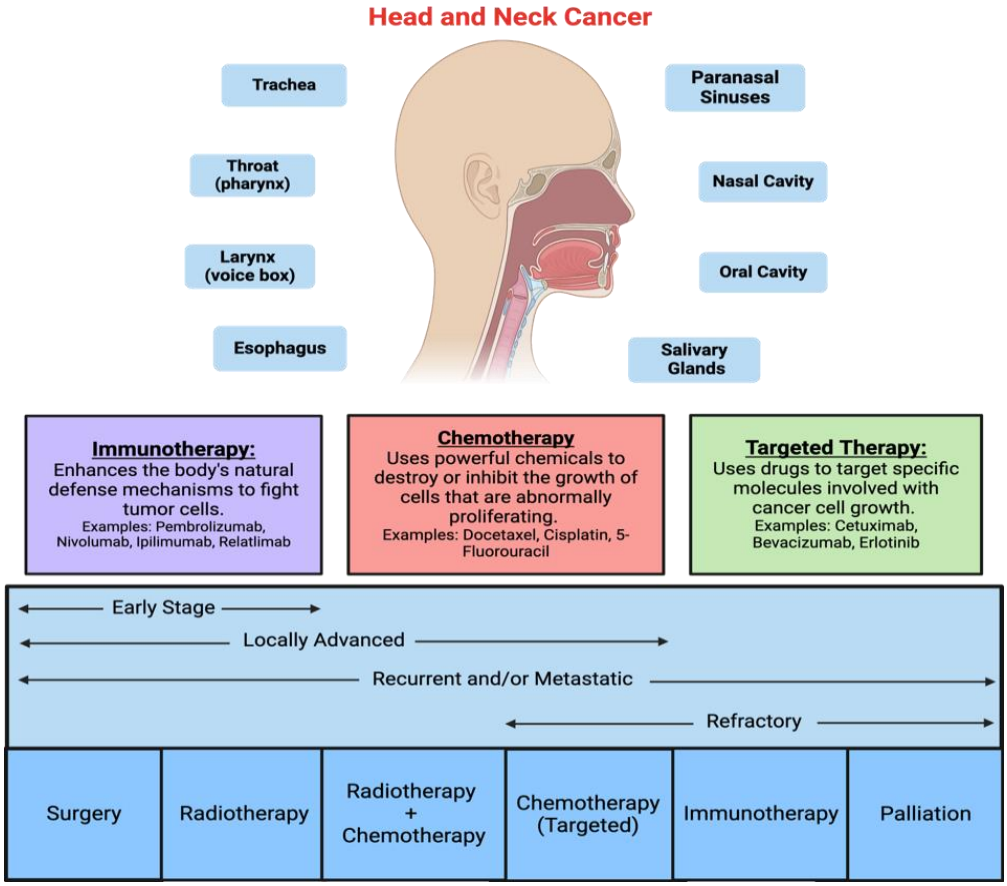

Figure 2. Overview of head and neck cancer. Head and neck cancer encompasses multiple cancer subtypes which originate in the epithelial cells lining the upper aerodigestive tract [61]. There are various methods of treating HNC depending on tumor stage, severity, and location [4], [62]. This figure was created using Biorender.com and published in the International Journal of Molecular Sciences [63].

The common signs of HNC include fatigue, sudden weight loss, loss of appetite, insomnia, and pain [64]. In addition, HNC patients often experience lymphadenopathy, dysphagia, recurrent aphthous stomatitis, dysphonia, and pharyngitis [61]. However, many of these symptoms are linked with other conditions that do not require primary medical care. Therefore, approximately 73% of HNCs are diagnosed at later stages of disease progression [65]. HNC is staged in the clinic using the TNM staging guidelines of the American Joint Committee on Cancer [66]. As is the case with multiple cancer types, the mortality rate of HNC significantly increases with advanced disease stage [67].

The standard treatment for HNC involves surgery, radiation therapy, and chemotherapy [4] (**Figure 2**). However, immunotherapy has recently been approved for the treatment of recurrent

and/or metastatic HNC (**Figure 2**) [62]. Understanding the risk factors associated with diseases allows us to better mitigate them and create preventative strategies to decrease harmful exposure [68], [69]. If prevention is not possible, understanding the specific driver of a malignancy allows clinicians to personalize treatment regimens and improve patient outcomes in the clinical setting. Depending on the severity of the disease, some patients will require a multimodal treatment regimen (**Figure 2**) [4].

2.4 Descriptive Epidemiology of Head and Neck Cancer

In 2020, GLOBOCAN estimated that 890,000 new cases of HNC are diagnosed around the world each year [50]. Unfortunately, approximately 450,000 people die from this condition annually [50]. The majority (70-80%) of HNC tumors originate in the oral cavity and can be attributed to tobacco and alcohol consumption [70]. Studies have found that smokers are up to ten times more likely to develop HNC throughout their lifetime [70]. Furthermore, infection with HPV accounts for an estimated 25% of all HNC cases [71].

Many high-income countries are experiencing an exponential increase in the incidence of oropharyngeal cancer (OPC) [72]. Within the last ten years, the incidence of HPV-related HNC has increased by approximately 36.5% [73]. Although global smoking prevalence is on the decline, the incidence of HPV-related HNC is increasing by approximately 2.5% each year [74], [75]. Epidemiologists have postulated that sexual behavior, non-monogamy, and a younger age of first sexual activity are related to the increased incidence of HPV-related HNC [76]. In addition, in recent years, the World Health Organization (WHO) has highlighted vaccine hesitancy as a major threat to global health [77]. Moreover, a study performed by Ryan *et al.* demonstrated that pediatric HPV vaccine hesitancy has increased by approximately 11% since the COVID-19 pandemic [78].

Global statistics have demonstrated that HNC is twice as common in men than in women [61]. This can be explained by differences in lifestyle and exposure to risk factors [79], [80], [81]. Furthermore, the median age of diagnosis of HPV-negative HNC patients is 66 years old, whereas the median age of diagnosis of HPV-positive HNC patients is 53 years old [61]. Therefore, HPV-related HNC is occurring in a significantly younger demographic [61]. In addition, HPV-related HNC has a five-year survival rate of 80%, whereas that of HPV-unrelated HNC is 40% [82], [83]. European age standardized HNC mortality rates in the United Kingdom have demonstrated that the mortality rate of HNC has increased by approximately 15% over the last decade [2].

2.5 Risk Factors for Head and Neck Cancer

The risk factors for HNC are often associated with lifestyle, genetics, environmental exposure, and socioeconomic status [84], [85], [86]. Studies have demonstrated that 70-80% of all HNC diagnoses are linked to smoking and excessive alcohol consumption [70], [87]. In this context, smoking includes cigarettes, chewing tobacco, cigars, betel quid, and electronic cigarettes [61]. Furthermore, the combination of these two lifestyle factors may potentialize HNC risk [86].

In recent decades, chronic infection with HPV has emerged as a significant risk factor for HNC [88]. However, there exists an HPV vaccine, Gardasil-9, which is effective at preventing infection against high-risk HPV subtypes [69]. This vaccine provides protection against nine common HPV strains (HPV-6, -11, -16, -18, -31, -33, -45, -52, and -58) [89]. Furthermore, inoculation with this vaccine provides cross-protection for additional HPV strains [90]. Unfortunately, despite preventive measures, current estimates demonstrate that up to 90% of OPC can be attributed to chronic infection with HPV [88]. Researchers postulate that the slow implementation of vaccination programs, reduced vaccination of males and minority groups, lack of HNC screening, and vaccine hesitancy contribute to the prevalence of HPV-related head and neck malignancies [91], [92], [93].

2.6 Human papillomavirus (HPV)

HPV is the most common sexually transmitted virus in the world, with an estimated 50-70% of all sexually active individuals becoming infected at some point in their lifetime [94]. HPV transmission occurs primarily via vaginal, anal, and oral sex [95]. The virus can also be transmitted via intimate skin-to-skin contact during intercourse [95]. An HPV infection can usually be cleared by the host's immune system [96]. However, in rare instances, the infection can persist and develop into benign or pre-malignant lesions [97]. The majority of individuals with persistent infection are asymptomatic [98]. Therefore, cancer screening is an important tool to monitor chronic HPV infections and eliminate pre-malignant lesions at early stages of development [99]. Unfortunately, there are currently no standardized screening methods for HNC [100], [101].

HPV is a non-enveloped DNA virus that is approximately 8,000 nucleotides in length [102]. At the present moment, over 200 HPV genotypes have been discovered [53]. HPV is

generally not considered a hereditary virus [103]. However, in rare instances, the virus can be passed between mother and offspring during pregnancy and childbirth [104]. Researchers postulate that this viral transmission is due to the direct contact of the fetus with the HPV-positive lesions in the anogenital tract of the mother [104]. Fortunately, children can spontaneously clear the virus without complications [104].

The various HPV strains are categorized based on the level of risk they pose to their human hosts [105]. High-risk HPV strains have the potential to cause cancer, whereas low-risk HPV strains have the potential to cause warts and other lesions [105], [106]. There currently exist 12 high-risk human papillomavirus strains which include HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and -59 [107]. The high-risk HPV strains most commonly cause a variety of anogenital cancers [108]. In fact, the vast majority of cervical (99.7%), anal (90%), penile (up to 50%), vulvar (up to 76.5%), and vaginal (74%) cancer can be attributed to chronic infection with HPV [108], [109], [110], [111], [112], [113]. In the United States of America, chronic HPV infection is the cause of 80% of OPC cases [114]. However, some HPV strains are more prevalent in certain geographic locations [115], [116]. For example, the most common HPV subtype in high income countries is HPV-16, whereas one of the most common HPV subtypes on the continent of Africa is HPV-35 [115], [116].

The genome of HPV encodes six early proteins (E1-E7) and two late proteins (L1-L2) (**Figure 3**) [117]. E1 and E2 are responsible for viral transcription and genome replication [117]. E4 is responsible for virion release, whereas E5 is a minor oncogene [117]. However, the major oncoproteins of HPV are E6 and E7 which, respectively, degrade and sequester p53 and Rb [117]. Notably, blocking the E6 oncoprotein using recombinant proteins, peptides, or antibodies, has been shown to drive growth arrest and/or death of HPV-positive cells [118], [119], [120], [121]. Finally, L1 and L2 constitute the capsid proteins which are responsible for viral assembly (**Figure 3**) [117].

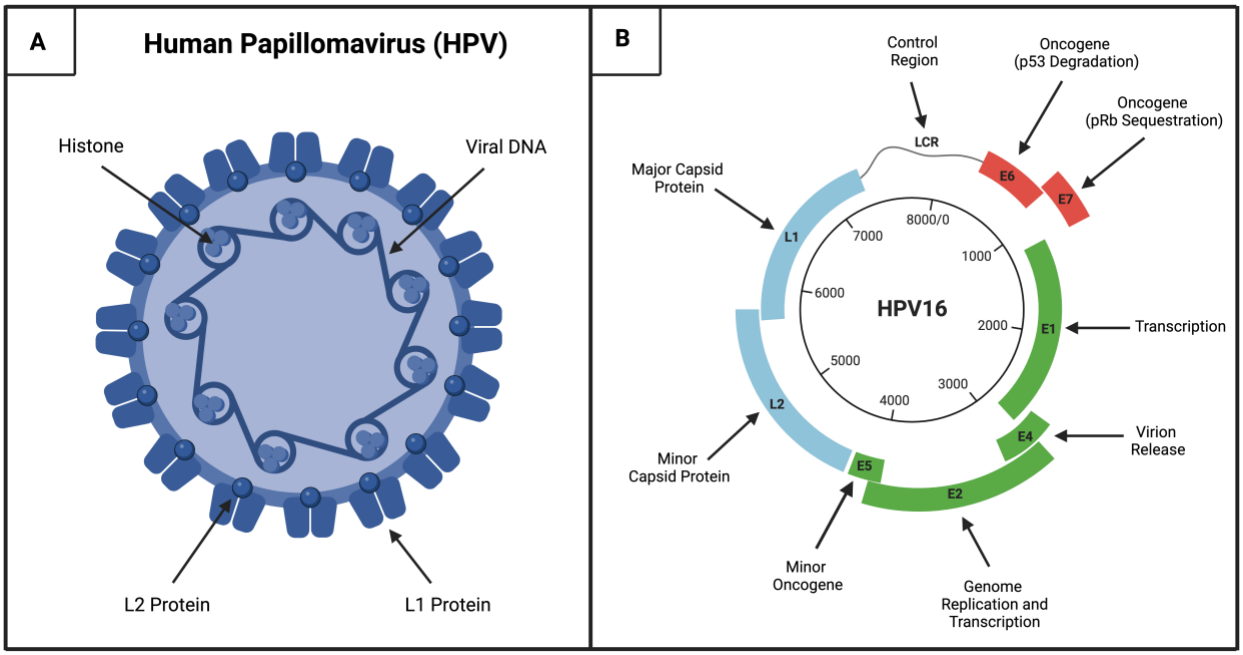

Figure 3. Human papillomavirus components. A. This panel showcases a macroscopic view of the HPV virus and its main components. The L1 and L2 proteins make up the viral capsid. The viral DNA and histones are contained within this viral capsid [117]. **B.** This panel showcases the different genes which make up the human papillomavirus [117]. HPV-16 is the most common cancer-causing HPV subtype in high-income countries [115]. Abbreviations: HPV, human papillomavirus; DNA, deoxyribonucleic acid; LCR, locus control region; pRb, retinoblastoma tumor suppressor protein; E1, early gene 1; E2, early gene 2; E4, early gene 4, E5, early gene 5, E6, early protein 6; E7, early protein 7, L1, late protein 1; L2, late protein 2. Figure created using Biorender.com.

The mechanism of HPV-induced carcinogenesis involves the oncoproteins E6 and E7 [117], [122]. Once the virus enters the host cell, oncoprotein E7 will bind and sequester the retinoblastoma protein (Rb) from its usual binding partner, E2F (**Figure 4C**) [117], [122]. This allows E2F, a potent transcriptional activator, to localize to the nucleus and upregulate the transcription of cell cycle genes, such as *CDKN2A* (**Figure 4C**) [117]. *CDKN2A* encodes p16, a tumor suppressor protein which compensates for the partial loss of Rb [123], [124]. p16 inhibits cyclin dependent kinases 4 and 6 (CDK4/6), thereby keeping Rb in its hypophosphorylated state so that it can remain bound to E2F and prevent progression through the cell cycle (**Figure 5**) [124], [125]. The expression of p16 in OPC has been linked with favourable patient prognosis [126], [127].

Furthermore, depending on the degree of cellular stress induced by the action of E7, the tumor suppressor protein p53 may direct the cell towards processes such as DNA repair, cellular senescence, or apoptosis. (**Figure 4D**) [117], [128]. The oncoprotein E6, in conjunction with the E6 associated protein (E6-AP), will then ubiquitinate p53 and target it for proteasomal degradation

(**Figure 4D**) [117], [122]. With the cellular stress sensor now degraded, the cell can undergo a malignant transformation [117].

The classification of certain HPV types as high-risk is based on their molecular characteristics, epidemiological associations with cancer, and clinical outcomes in infected individuals [129]. High-risk HPV subtypes are more carcinogenic since their oncoproteins have a higher affinity for their respective binding partners [130]. The amino acid sequence of the E7 oncoprotein has been highly conserved throughout evolution, whereas that of oncoprotein E6 varies depending on the HPV subtype [131]. Oncoproteins E6 and E7 assume a central role in the initiation of HNC, encompassing functions from the maintenance of proliferative signaling and the circumvention of tumor suppressors, to activating telomerase and inducing angiogenesis [132], [133], [134]. These functions, which align with the primary hallmarks of cancer established by Hanahan and Weinberg (2000), eventually culminate in invasion and the metastatic dissemination of cancer cells [46]. Remarkably, E6 and E7 individually orchestrate all six cancer hallmarks, facilitating the establishment and successful progression of HNC [119], [135], [136].

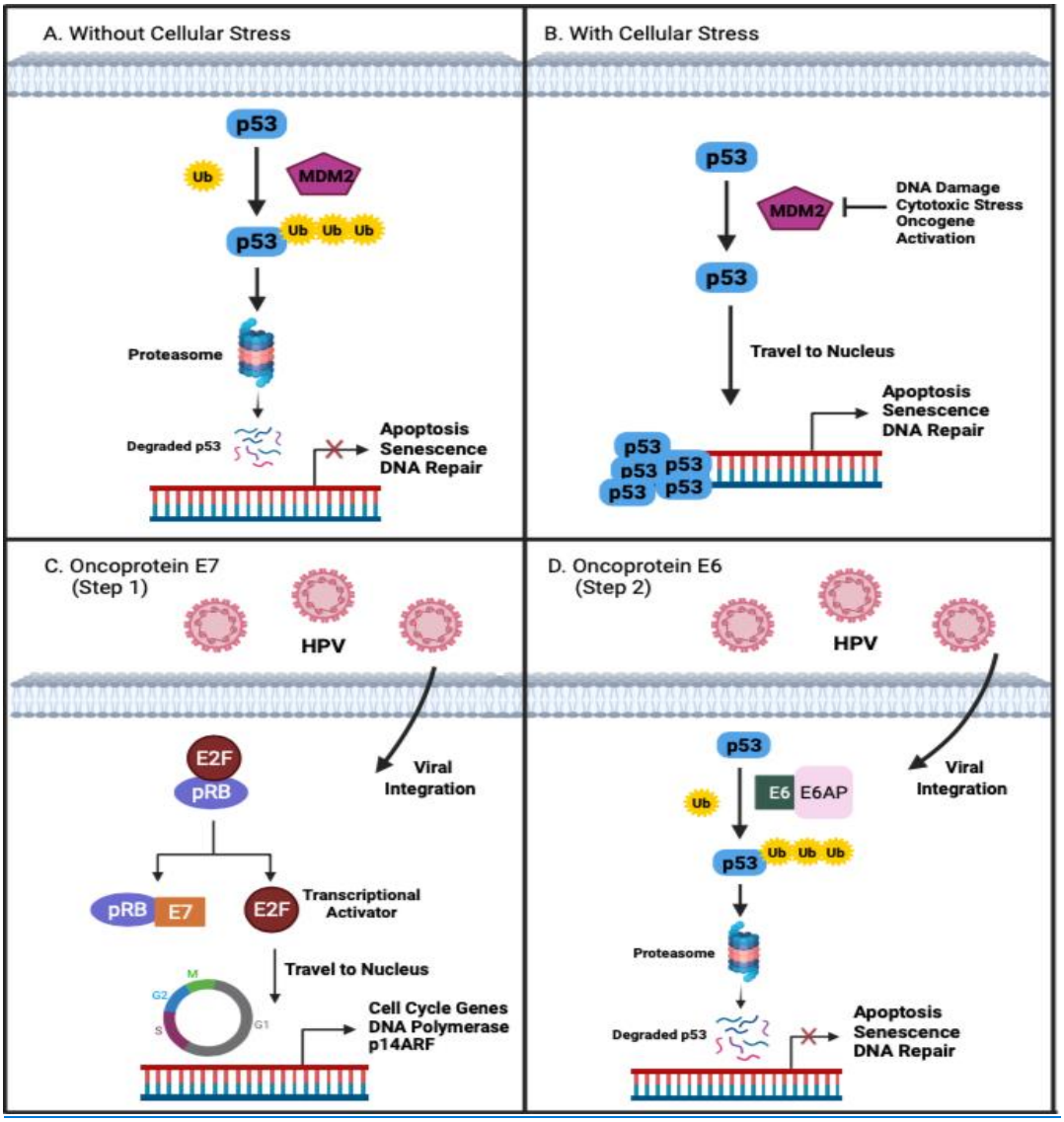

Figure 4. The cancer-causing mechanism of human papillomavirus. A. This panel demonstrates that under normal cellular conditions, p53 is negatively regulated by MDM2 and targeted for proteasomal degradation [137], [138]. **B.** This panel demonstrates that under conditions of cellular stress, MDM2 is inhibited [137], [138]. This allows p53 to travel to the nucleus and upregulate the transcription of apoptosis, senescence, or DNA repair genes [137], [138]. **C.** This panel demonstrates the first step of HPV-induced carcinogenesis which involves the sequestration of tumor suppressor protein Rb by oncoprotein E7 [117], [122]. **D.** This panel demonstrates the second step of HPV-induced carcinogenesis which involves the ubiquitination and subsequent proteasomal degradation of p53 [117], [122]. Abbreviations: MDM2, mouse double minute 2 homolog; Ub, ubiquitin; DNA, deoxyribonucleic acid; HPV, human papillomavirus; pRB, retinoblastoma tumor suppressor protein; E2F, family of transcription factors; p14ARF, alternate reading frame of the CDKN2A locus; E6AP, E6 associated protein; G1, gap 1 phase of the cell cycle; S, synthesis phase of the cell cycle; G2, gap 2 phase of the cell cycle; M; mitosis. Figure created using Biorender.com.

2.7 HPV-Related Head and Neck Cancer

HPV-related HNC is a molecularly and clinically distinct HNC subtype (**Table 1**). HPV-related HNC mainly occurs in the oropharynx (up to 80%), a region which encompasses the tonsils,

the base of the tongue, and the soft palate [61], [139]. Researchers have postulated that this region is most commonly affected since the tonsillar crypts have deep invaginations that serve as a site for the accumulation of foreign bodies [140]. Furthermore, the oropharynx contains mucosal-associated lymphoid tissue (MALT) which contributes to the specialized immune microenvironment in this region [141]. However, despite the specialized microenvironment in the oropharynx, HPV can circumvent detection and clearance mechanisms, leading to persistent infection [141]. In addition, PD-L1 expression is heightened in the tonsillar region which allows viruses and bacteria to evade the immune system [140]. HPV-positive HNC patients also have a smaller mutational load [142]. Therefore, their malignant cells are more susceptible to pro-apoptotic agents that exploit tumor suppressor properties [142]. HPV-unrelated HNC is characterized by a higher frequency of mutations in tumor suppressor genes like *TP53* and *CDKN2A,* as well as alterations in oncogenes such as *PIK3CA* and *EGFR* [143]. Despite these many differences, HPV-positive and HPV-negative HNC are currently managed with similar drug combinations in the clinic.

Table 1. Differences between HPV-related and HPV-unrelated head and neck cancer.

Parameters	HPV-Related HNC	HPV-Unrelated HNC
Median age of diagnosis	53	66
Most common risk factors	Infection with HPV	Tobacco and alcohol
Site of origin	Oropharynx	Oral cavity and larynx
5-year survival	80%	40%
Most affected population	Caucasians	Asian and African Americans
Mutational burden	Low	High
Disease onset	Integration of HPV into the genome (action of E6 and E7)	TP53, Rb, HRAS, EGFR, CASP8, PIK3CA, and RTK mutations
Response to anti-cancer treatment	Good response	Poor response
Differentiation status	Poorly differentiated	Well differentiated
Immunogenicity	High	Low

Abbreviations: HPV, human papillomavirus; HNC, head and neck cancer; Rb, retinoblastoma tumor suppressor protein; HRAS, Harvey rat sarcoma virus; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase; RTK, receptor tyrosine kinase. Sources: [86], [144], [145], [146], [147], [148], [149], [150], [151], [152].

Researchers use p16 immunohistochemistry (IHC), DNA *in situ* hybridization, and RNA *in situ* hybridization for assessing the HPV status of HNC patients [153], [154], [155]. However, the most common method for diagnosing HPV positivity in the clinic is via p16 IHC analysis [156]. IHC is a laboratory method that uses the principles of antibody recognition to detect antigens

of interest in a tissue sample (**Figure 5**) [157]. The antibody is linked to a reporter molecule that releases a colored pigment when an antigen-antibody reaction occurs [157]. This pigment can stain the tissue and then be visualized via light microscopy (**Figure 5**) [157]. p16 is a globally accepted surrogate marker for assessing HPV positivity, however, it is not 100% accurate in the diagnosis of HPV-related OPC [156]. Multiple studies have demonstrated that p16 IHC has a false positivity rate of approximately 15% in the clinic [126]. This can be attributed to the fact that somatic mutations of the Rb protein and excessive cell population doublings contribute to p16 expression [158]. This is contrary to HPV-unrelated HNC, in which p16 expression in tumor tissue is significantly decreased [140]. Relying on a surrogate marker with a high false positivity rate could have negative implications on treatment decision-making processes [159]. In the case of HNC, misdiagnosing the viral status of a patient could lead to the inappropriate recommendation for a de-escalated treatment regimen [159]. Furthermore, variability in p16 staining protocols, interpretation criteria, and scoring systems across laboratories can impact the consistency and reproducibility of results [160], [161], [162]. Standardization of testing methods and interpretation guidelines is essential for ensuring an accurate and reliable p16 assessment [162].

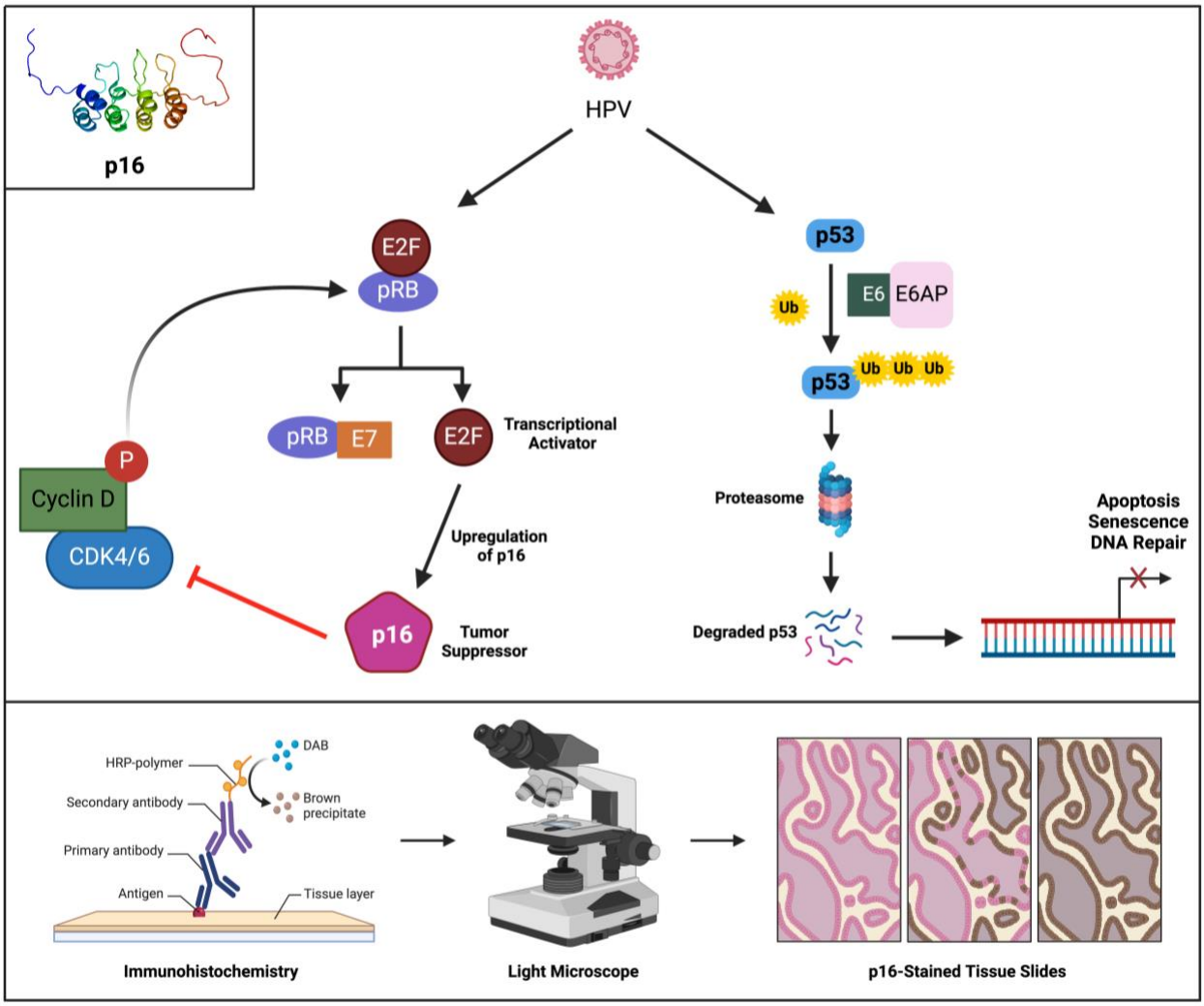

Figure 5. HPV infection and the subsequent increase in p16 expression. When the transcriptional activator E2F is released from pRb, the levels of tumor suppressor p16 increase in the cell [117]. This cellular mechanism has evolved to compensate for the partial loss of the Rb tumor suppressor protein [123], [124]. p16 will inhibit CDK4/6, thereby preventing the Rb protein from being further phosphorylated [124], [125]. p16 is a surrogate marker for diagnosing HPV positivity in the clinic and is detected via immunohistochemistry antibody staining [156]. Abbreviations: HPV, human papillomavirus; E2F, family of transcription factors; pRB, retinoblastoma tumor suppressor protein; P, phosphate; CDK4/6, cyclin-dependent kinase 4 and 6; Ub, ubiquitin; E6AP, E6 associated protein; DNA, deoxyribonucleic acid; DAB, diaminobenzidine; HRP-polymer, horseradish peroxidase-polymer. Figure created using Biorender.com.

2.8 The Tumor Microenvironment

In recent decades, the roles of the TME on cancer development and progression have been explored [163]. The TME refers to the heterogeneous cellular and non-cellular components surrounding the tumor mass which play a crucial role in tumor development, progression, and response to therapy [164]. The TME includes stromal cells such as fibroblasts, endothelial cells, adipocytes, and ECM components [164]. The TME also contains immune cells such as tumor

infiltrating lymphocytes (TILs), tumor associated macrophages (TAM), myeloid-derived suppressor cells (MDSCs), natural killer cells (NKs), regulatory T cells (Treg), dendritic cells (DCs), and B cells [164], [165]. Furthermore, the TME can secrete or recruit factors that enable the formation of a pre-metastatic niche [164]. When cancer cells disseminate from a primary tumor, they will be recruited to permissive environments to form metastatic colonies in distant organs [164].

Malignant cells have the capacity to modify immune microenvironments [164]. Immune cells express cytokines, growth factors, and proteolytic enzymes (e.g., matrix metalloproteinases (MMP), tumor necrosis factor-alpha (TNF-α), interleukins (ILs), vascular endothelial growth factor (VEGF), and transforming growth factor-beta (TGFβ)) which can influence metabolic changes and alter the phenotype of the TME [140], [166]. In this context, both the innate immune system and the adaptive immune system play key roles in surveillance against the initiation, development, and progression of HNC [167]. When the immune system is impaired, cancer cells can proliferate in an uncontrolled manner [168]. Unfortunately, tumor cells can adapt several mechanisms to escape immune surveillance and promote tumor cell proliferation, survival, and metastasis [169].

Recent advancements, such as the refinement of single cell sequencing and omics' technologies, have facilitated our understanding of the complex TME [170]. There is abundant crosstalk between cancer cells and the cells within the microenvironment [167]. For example, neoplastic cells secrete factors into their surroundings which can promote the acquisition of certain cancer hallmarks, such as angiogenesis, invasion, and metastasis [171], [172]. Furthermore, TAMs can remodel the extracellular matrix, as well as recruit immunosuppressive cells [173], [174]. The dynamic evolution of the TME, the interplay between immune and non-immune cells, and the mechanisms of immune evasion are commonly exploited in order to develop advanced therapeutics [170].

2.9 Current Treatment Strategies

To provide comprehensive cancer care tailored to an individual HNC patient's needs, a multidisciplinary team is often needed [175]. The standard treatment for HNC consists of surgery followed by chemotherapy and/or radiation therapy [4]. In recent years, immunotherapy and targeted therapy have also been implemented when a primary course of cancer treatment is

unsuccessful [176]. The main goal of surgery is to remove a tumor in its entirety, however, some malignant cells may remain [4]. Therefore, surgery is often followed by an additional anti-cancer treatment to assure that all malignant cells are eliminated [4].

Radiation therapy, which is the second most common HNC treatment, uses high-energy radiation to eliminate or shrink a tumor [177]. Radiation therapy for HNC includes two main types: external beam radiation therapy (EBRT) and internal radiation therapy (Brachytherapy) [178], [179]. EBRT involves using high-energy beams, generated by a machine called a linear accelerator, to target the tumor from outside of the body [180]. In addition, techniques like intensity-modulated radiation therapy (IMRT) and image-guided radiation therapy (IGRT) enhance precision and minimize damage to surrounding healthy tissue [180], [181]. Brachytherapy, on the other hand, involves placing radioactive sources directly within or near the tumor, thereby delivering high doses of radiation to the cancer cells while sparing nearby healthy tissue [180]. Both radiation methods are tailored to tumor size, location, and stage, and offer effective treatment options for patients with HNC [182]. Radiation therapy for HNC can cause various side effects, both acute and chronic [183]. Acute side effects, which occur during or shortly after treatment, include skin irritation, xerostomia, changes in taste, and fatigue [183]. Chronic side effects, which may develop years later, can include persistent xerostomia, dental problems, tissue scarring, lymphedema, hypothyroidism, and changes in voice quality [183].

Chemotherapy is another common HNC treatment which involves the intravenous administration of cytotoxic drugs [184]. Currently, multiple chemotherapy drugs, such as cisplatin, carboplatin, 5-fluorouracil (5-FU), docetaxel (Taxotere), and paclitaxel (Taxol), are approved by the FDA (Food and Drug Administration) to treat HNC patients [184]. Chemotherapy drugs can be administered as a primary treatment, in combination with other therapies (adjuvant, neoadjuvant, and concurrent therapy), or for palliative care [185], [186]. Platinum chemotherapy (cisplatin) is most commonly used in the context of HNC [184], [187]. The side effects of chemotherapy are often acute and involve fatigue, nausea, hair loss, infection, as well as liver damage [188], [189]. Both chemotherapy and radiation therapy prevent the growth of rapidly proliferating cells in the body [190]. Therefore, careful administration and management of the side effects for both treatment modalities is necessary to optimize outcomes and maintain patient quality of life.

Immunotherapy, which is an innovative HNC treatment, involves boosting a patient's immune system for it to better target cancer cells [176]. This treatment modality has shown promise, particularly in cases where traditional treatments like surgery, radiation, and chemotherapy are less effective [191]. Immunotherapy approaches often involve checkpoint inhibitors which block the action of immunosuppressive proteins in order to enhance the immune system's ability to attack neoplastic cells [191], [192], [193]. In addition, a patient's immune cells, notably their T cells, can be engineered via adoptive cell therapy (CAR-T) in order to better recognize and destroy cancer cells [191], [194], [195]. Furthermore, cancer vaccines which stimulate the immune system to fight cancer encompass another immunotherapy approach [191], [196]. Pembrolizumab and nivolumab are the only immunotherapy drugs currently approved for the treatment of recurrent or metastatic HNC [176]. These drugs inhibit the interaction of PD-1 and PD-L1, thereby allowing T cells to become properly activated and subsequently perform their immune surveillance roles [176]. Immunotherapy also comes with unique side effects and challenges, especially those related to auto-immune reactivity [197]. Patients receiving immunotherapy require careful monitoring to manage side effects and assess treatment efficacy [197].

Lastly, targeted therapy is a specialized treatment approach which selectively targets molecules involved in cancer cell growth and survival [198]. By targeting specific pathways and proteins involved in carcinogenesis, targeted therapies can be highly effective, more precise than traditional chemotherapy, and have fewer side effects [198], [199]. Ongoing research continues to refine targeted therapies and expand their use in clinical practice. Currently, cetuximab is the only FDA approved targeted therapy drug to treat HNC [199]. Cetuximab is a monoclonal antibody drug which targets the extracellular domain of the epidermal growth factor receptor (EGFR), thereby preventing its ligand from binding [199]. The blocking of this interaction impacts angiogenesis, apoptosis, cell cycle progression, and metastasis [199]. In addition, studies have shown that the administration of cetuximab enhances a patient's response to chemoradiation therapy [199].

Due to the favorable prognosis of HPV-positive HNC patients, clinicians and researchers have recently proposed clinical trials to de-escalate treatment, limit toxicities, and reduce chronic side effects [82], [83], [200]. One such treatment is NECTORs, which is an acronym for neo-adjuvant chemotherapy followed by transoral surgery and selective neck dissection [201].

Additional de-escalated treatment strategies have been proposed in several clinical trials for HPV-positive HNC, such as reducing the dose of radiation or standard drugs used in the clinic (clinicaltrials.gov-NCT01932697, NCT00606294, NCT01530997) [200]. These trials aim to identify more effective therapies, improve patient outcomes, and expand the understanding of HNC [200].

2.10 Liquid Biopsy

A liquid biopsy is a minimally invasive technology that allows us to isolate analytes of interest from a variety of biofluids including blood, saliva, urine, and pleural effusion (**Figure 6**) [202]. Analytes of interest include circulating tumor cells (CTC), circulating tumor DNA (ctDNA), tumor proteins, exosomes, and methylation changes (**Figure 6**) [202]. Traditional biopsies are invasive, non-repeatable, and often impossible in hard-to-reach areas of the body [202]. Liquid biopsies, on the other hand, are minimally invasive, repeatable, and can comprehensively assess tumors in precarious locations (**Figure 6**) [202]. Furthermore, liquid biopsies have the potential to greatly reduce the burden on patients and the health care system. Liquid biopsies have applications in cancer detection, assessing minimal residual disease, predicting patient prognosis, and predicting therapeutic resistance (**Figure 6**) [203].

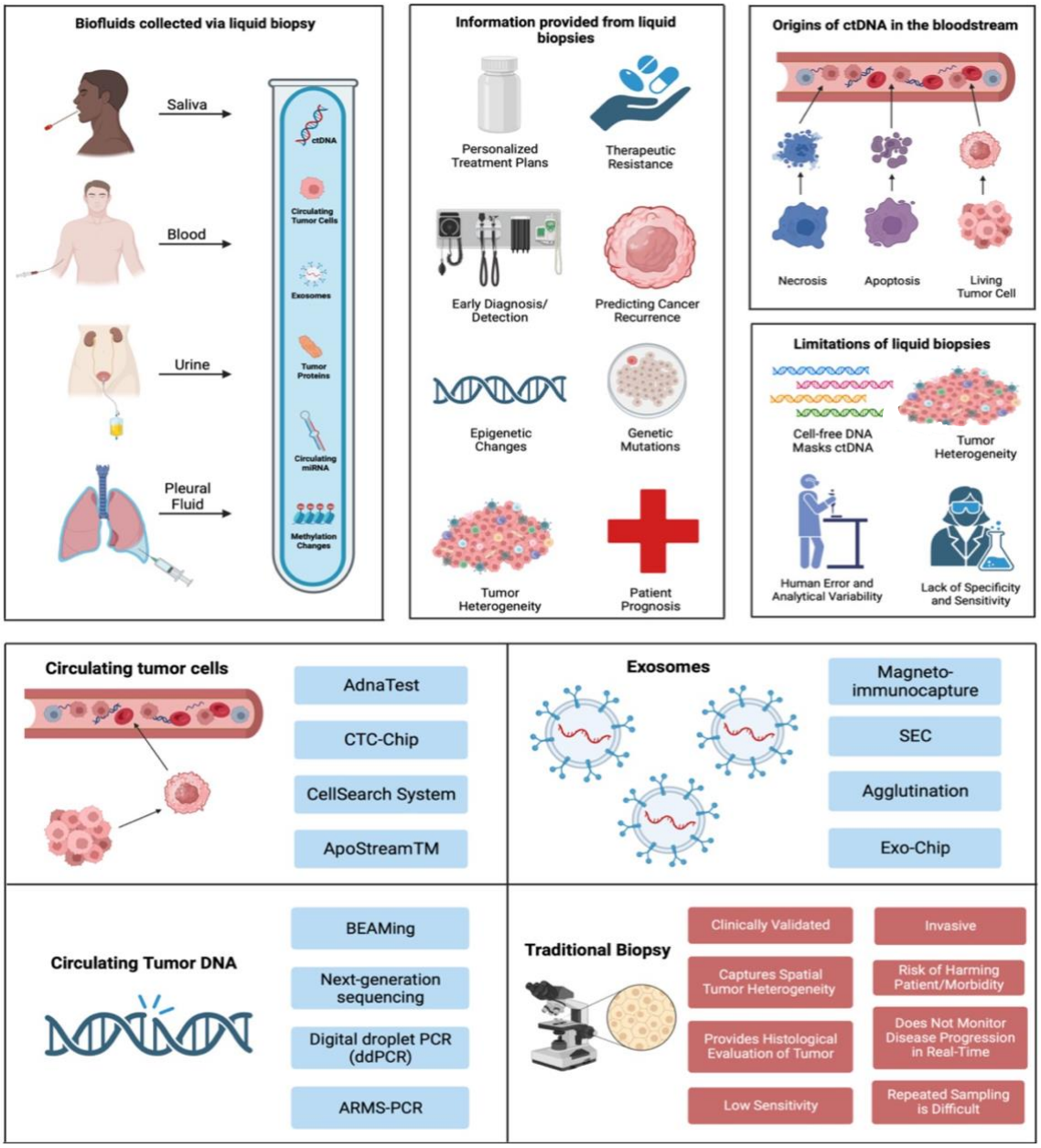

Figure 6. Applications and characteristics of liquid biopsies. Liquid biopsies can be performed using a variety of biofluids such as saliva, blood, urine, and pleural fluid [202]. Liquid biopsies allow researchers to analyze circulating tumor cells, circulating tumor DNA, exosomes, tumor proteins, circulating microRNAs, and methylation changes [202]. Liquid biopsies have applications in cancer diagnosis, predicting cancer recurrence, predicting patient prognosis, and more [203], [204]. Many liquid biopsy methods currently exist as this technology is not yet standardized. Abbreviations: ctDNA, circulating tumor deoxyribonucleic acids; circulating miRNA, circulating micro ribonucleic acids; CTC-Chip, circulating tumor cell chromatin immunoprecipitation; BEAMing, polymerase chain reaction involving beads, emulsion, amplification, and magnetics; ARMS-PCR, amplification refractory mutation system-polymerase chain reaction; SEC, size exclusion chromatography; Exo-Chip, chromatin immunoprecipitation with exonuclease treatment. Figure created using Biorender.com.

Liquid biopsy technology is not yet clinically validated in the context in HNC. However, this technology has shown promise in non-small cell lung cancer (NSCLC), breast cancer, ovarian

cancer, and prostate cancer [205]. Researchers have hopes that liquid biopsy technology will greatly improve the prognosis of cancer patients by allowing them to be treated earlier and more effectively. Once liquid biopsies become standardized, their use as a complementary tool in the clinical setting will be widespread. The manuscript in the following chapter will explore the applications and limitations of liquid biopsies in the context of HNC.

CHAPTER 3 – MANUSCRIPT 1

CURRENT STATUS OF CIRCULATING TUMOR DNA AND CELL CIRCULATING ALTERATIONS IN HEAD AND NECK CANCER

A literature and systematic review

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3.1 ABSTRACT

Head and neck cancer (HNC) is the sixth most prevalent cancer worldwide with a poor prognosis when diagnosed at advanced clinical stages. The main risk factors are tobacco consumption and alcohol abuse. However, the incidence of oropharyngeal cancer (OPC) is increasing due to human papillomavirus (HPV) infection. Current diagnostic techniques for both HPV-positive and HPV-negative HNC often involve invasive, costly, and time intensive procedures. Alternatively, liquid biopsies have emerged as a minimally invasive technique which may lessen the burden of cancer diagnoses on both patients and healthcare resources. This technique analyzes biological components released by tumors into the bloodstream, such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), exosomes, tumor proteins, and methylation changes, allowing for specific cancer detection and surveillance. This article reviewed the status and clinical applications of ctDNA and CTCs in the diagnosis and treatment of HPV-positive HNC. In addition, a systematic review (PROSPERO ID: 560498) was conducted to investigate whether liquid biopsies could be leveraged to assess the role of the immune system on treatment outcomes and the overall survival of HNC patients. Two public databases (Medline and Embase) were searched using relevant MeSH (Medical Subject Headings) terms and keywords. After multiple rounds of screening, eight studies published between 2017 and 2024 involving 814 cancer patients from three different countries were retained for data extraction. The data demonstrated that the immune microenvironment of HNC patients could be characterized via liquid biopsy, however, future validation is required. Furthermore, through the detection of HPV ctDNA, liquid biopsy technology has shown promise in diagnostics, as a predictor of patient prognoses and treatment responses, and as a tool to monitor disease progression in HPV-positive HNC.

KEYWORDS: Head and neck cancer, human papillomavirus, oropharyngeal cancer, liquid biopsy, prognosis, circulating tumor cells, circulating tumor DNA, immune microenvironment.

3.2 INTRODUCTION

Head and neck cancer (HNC) is the sixth most prevalent cancer around the world with more than 660,000 new cases and 325,000 deaths per year [1]. Cancers of the head and neck region encompass all malignancies originating in the upper aerodigestive tract, including the oral cavity, oropharynx, larynx, paranasal sinuses, and nasal cavity (**Figure 7**) [2]. Common risk factors include smoking, alcohol abuse, and human papillomavirus (HPV) infection (**Figure 7**) [3]. The standard of care for HNC is similar regardless of tumor HPV status and often consists of surgery [4], followed by radiotherapy, chemoradiation therapy, or multimodal treatment regimens depending on the clinical stage of the disease [5], [6]. These treatments are often accompanied by adverse effects such as dysphagia, xerostomia, dysarthria, among others [7], which can severely impair patient quality of life. As HPV-positive HNC patients have an improved response to chemoradiotherapy and superior survival rates in comparison to HPV-negative HNC patients [5], clinical trials have been proposed to de-escalate therapies in these patients (**Figure 7**). De-escalated therapeutic approaches include minimally invasive surgery (e.g., transoral robotic surgery (TORS)), reduced dosage radiotherapy, targeted therapy (e.g., EGFR and VEGF inhibitors), and immunotherapy (e.g., anti-PD1/-PDL1, anti-CTL4) (**Figure 7**) [8]. These approaches aim to improve patient outcomes by reducing treatment-related toxicities, while delivering effective treatment [8]. However, precise diagnoses are imperative to optimize outcomes.

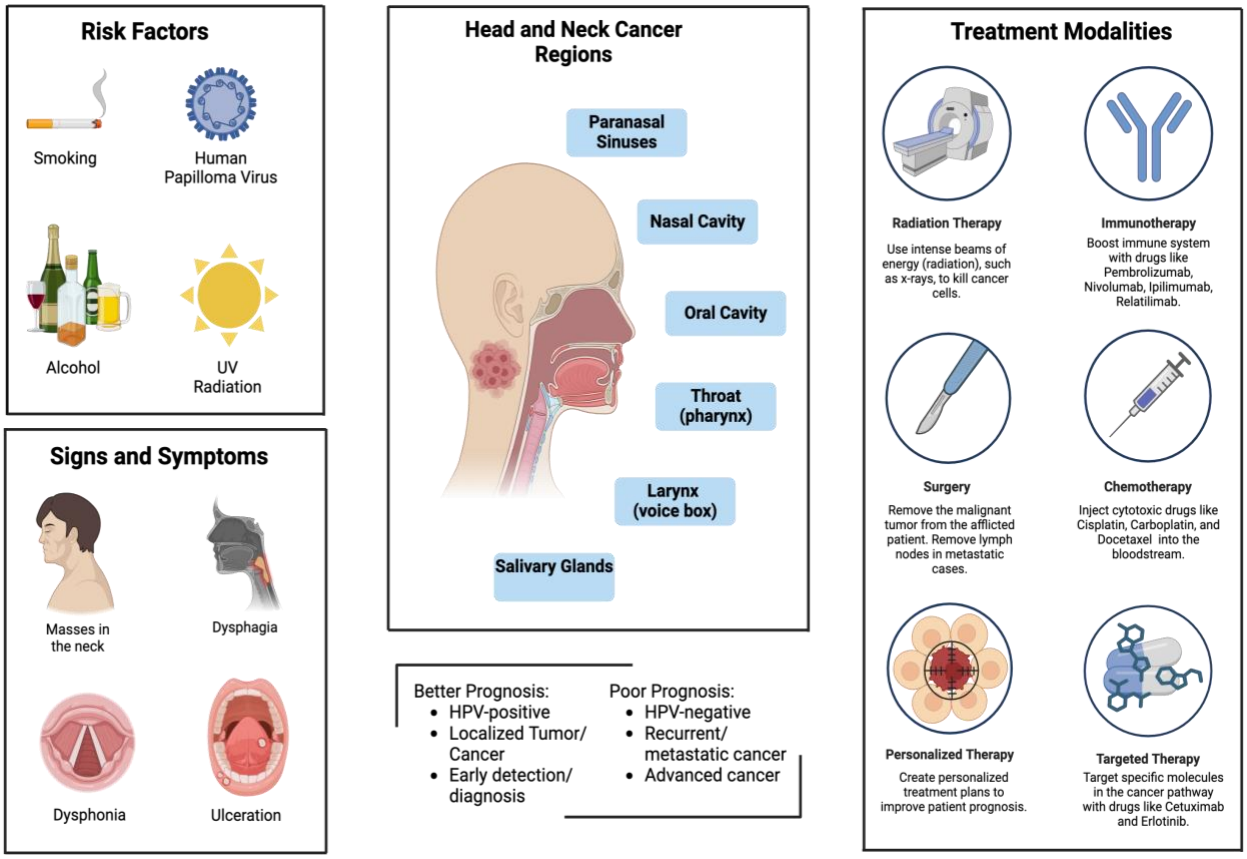

Figure 7. Common risk factors, clinical characteristics, and treatment modalities of head and neck cancer (HNC). HNC can occur in the oral cavity, oropharynx, larynx, paranasal sinuses, and nasal cavity [2]. Major risk factors associated with the development of HNC include smoking, alcohol abuse, and HPV infection [3]. The most common symptoms include masses in the neck, dysphagia, trismus, dysphonia and ulceration [7]. The standard of care consists of a combination of surgery, radiation therapy, and chemotherapy [4]. Personalized and targeted therapies, as well as immunotherapies, have emerged as new treatment strategies. Abbreviations: HPV, human papillomavirus; HPV-positive, human papillomavirus-positive; HPV-negative, human papillomavirus-negative. Figure created using Biorender.com.

HNC diagnosis currently relies on imaging procedures such as magnetic resonance imaging (MRI), computed tomography (CT) scans, and positron emission tomography/computed tomography scans (PET/CT scans), complemented by histopathological analysis [9]. Due to the prevalence of HPV infections in oropharyngeal cancer (OPC), HPV testing using polymerase chain reaction (PCR), *in situ* hybridization (ISH), or p16 immunohistochemistry (IHC) staining as a surrogate marker for viral infection has been recommended for this HNC subtype [10]. However, while conventional tissue-based diagnostic methods are effective, they are invasive and require long processing times, tissue preparation, and histological analysis [11]. Alternatively, liquid biopsies have emerged as a novel, less-invasive technique allowing for the isolation and analysis of biological components released by tumors in blood, saliva, urine, or other biofluids [11].

Particularly, liquid biopsies allow for the detection and analysis of circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), exosomes, tumor proteins, and methylation changes, which have shown to be promising biomarkers in both HPV-positive and -negative HNC [11]. Applications of liquid biopsies include assessing tumor heterogeneity, predicting immune checkpoint blockade responses, assessing inaccessible tumors, early cancer detection, predicting patient prognosis, evaluating treatment response and resistance, and assessing minimal residual disease (MRD) [12]. Despite these applications, this relatively novel technology is not yet clinically validated in the context of HNC, though multiple clinical trials are underway [13]. With emerging evidence on the utility of liquid biopsies in HNC, particularly in HPV-positive HNC, this study will explore their uses through two avenues: a literature review investigating their potential applications and limitations in the screening, diagnosis, prognostic assessment, and treatment of HPV-positive HNC, and a systematic review assessing whether this technique can be used to leverage the immune microenvironments of HNC patients.

3.3 METHODOLOGY

The findings of the systematic review are reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guidelines. The complete protocol for the systematic review is pending approval in PROSPERO (ID: 560498).

3.3.1 Information sources

3.3.1.1 Narrative literature review

Three public databases (Medline, PubMed, Google Scholar) were searched for articles pertaining to the applications of liquid biopsies in HPV-positive HNC.

3.3.1.2 Systematic review

A senior medical librarian searched through the Medline (Ovid) and Embase (Ovid) databases from inception until June 20, 2024. The search strategy used multiple key words found in the title, abstract, keyword fields, and relevant subject headings to retrieve articles looking broadly at the use of liquid biopsies to characterize the immune systems of cancer patients, with no language restriction. The full search strategy for Embase (Ovid) and the PRISMA 2020 checklist are

described in the supplementary material. The characteristics of the included studies are detailed in **Table S1**.

3.3.2 Inclusion criteria

3.3.2.1 Narrative literature review

The inclusion criteria considered bodily fluid collected (blood or saliva), patients diagnosed with HNC, HPV-positive status, early diagnostic potential, patient prognosis, disease recurrence, and treatment response.

3.3.2.2 Systematic review

The following inclusion criteria were considered: liquid biopsy analyte collected (CTC, ctDNA, and cell-free DNA (cfDNA)), study design (cohort studies, clinical trials, cross-sectional studies, pilot studies, case studies, case-control studies), patients diagnosed with HNC, patient survival, treatment response, and the immune microenvironment.

3.3.3 Exclusion criteria

3.3.3.1 Narrative literature review

All articles that did not focus on CTCs, ctDNA, and cfDNA were excluded from the literature review. This includes articles that focused on DNA methylation, tumor proteins, exosomes, and miRNA. Furthermore, articles that focused on HPV-negative HNC were also excluded from the study.

3.3.3.2 Systematic review

The following exclusion criteria were considered: articles written in a language other than English, sample size not reported, articles using auxiliary liquid biopsy analytes, unavailable articles, review articles, articles focusing on cancer types other than HNC, and the analysis of markers that are not related to the immune system.

3.3.4 Study selection

3.3.4.1 Narrative literature review

The titles and abstracts of articles of interest were manually screened and assessed for relevance based on the inclusion criteria. If the articles passed the initial screening, the main body of the text

was read, and the article was once again assessed for eligibility. No systematic protocol was followed for the narrative literature review.

3.3.4.2 Systematic review

The titles and abstracts of the identified studies were screened on Covidence by an independent reviewer (MA) for relevance based on the inclusion criteria. If the articles passed the initial screening, the main body of the text was read, and the article was once again assessed for eligibility. Any disagreements were resolved through discussion or consultation with two additional reviewers (FF and SDS). These additional reviewers took part in the final acceptance of the articles in the systematic review. The primary reasons for article exclusion were documented in Excel (Microsoft Office 365, Windows).

3.3.5 Data extraction

3.3.5.1 Narrative literature review

Data extraction for the narrative literature review was mainly qualitative. However, quantitative results, such as assay sensitivity percentages and survival metrics, were also collected.

3.3.5.2 Systematic review

The data from the eligible articles was extracted independently by two authors (MA and FF) using a standardized data extraction form in Excel (Microsoft Office 365, Windows) (**Table S1**). Extracted information included author, year of publication, country of study, study design, and number of participants. In addition, the liquid biopsy analyte used in the study and any key findings were also collected. Risk of bias (RoB) assessments were conducted using CASP (Critical Appraisal Skills Programme) checklists for each of the articles included in the systematic review.

3.4 RESULTS

3.4.1 Liquid biopsies as novel diagnostic tools

Due to advancements in omics' technologies, liquid biopsies have become promising diagnostic tools that could address the limitations of tissue biopsies by capturing genetic changes over time [14]. Tissue-based tumor profiles are often limited by sampling bias, offer a snapshot of tumor heterogeneity, and cannot be obtained repeatedly without invasive procedures [15]. In contrast, the

analysis of circulating genetic and cellular biomarkers in biofluids presents a minimally invasive avenue for cancer diagnosis [11]. In HNC, CTCs and ctDNA have shown promise as indicators of active disease, particularly among HPV-positive patients [16], [17].

CTCs are viable tumor cells that have been shed from a primary tumor or metastatic site into the bloodstream or other biofluids [18]. Although most CTCs have a short lifespan once in the circulation, certain tumor cells can survive in the harsh circulatory environment, extravasate into distant organs, and begin creating metastatic colonies [18]. Though research remains limited, studies have shown that the presence of CTCs has been correlated with poor patient survival rates [19], reduced treatment responses [20], late-stage disease [19], [20], and nodal involvement in HPV-positive and -negative HNC [19], [21]. Similarly, ctDNA consists of small fragments of DNA released into the bloodstream by viable, apoptotic, or necrotic tumor cells [22]. These fragments contain genetic and molecular information that can be used to characterize the tumor, such as gene mutations, epigenetic patterns, and copy number variations [22]. Both CTCs and ctDNA provide complementary insights into tumor biology: while CTCs can give information on the viability and metastatic potential of cancer cells [23], ctDNA offers a snapshot of the genetic alterations present in the tumor [24].

Though evidence supporting the use of CTCs as a diagnostic method in HNC is limited, the combination of CTC and ctDNA analysis enhances the understanding of tumor heterogeneity and the genetic landscape, making them promising tools for the diagnosis, monitoring, and treatment of cancer. For instance, HPV ctDNA was found to be significantly more accurate in the diagnosis of HPV-positive HNC, with a sensitivity of 98.6% compared to 72% with tissue biopsies and histological HPV testing [25]. Increased accuracies of HPV ctDNA detection compared to standard diagnostic imaging were reported in several studies [26], [27], [28]. The use of ctDNA as a diagnostic biomarker is further corroborated by sensitivities, specificities, and positive and negative predictive values above 90% [26], [27], [28]. Through its detection, ctDNA might provide an avenue for early HNC screening. A study by Rettig *et al*. showed that HPV ctDNA could be detected in plasma samples around 30.5 months before diagnosis, without false positives [29]. Further analyses in a secondary cohort showed that HPV ctDNA was detected in the pre-diagnostic plasma of 43% of HPV-positive OPC patients [29]. Though promising, conclusions from these findings must be drawn with caution as these studies had small samples sizes and require further validation.

Despite their promise, the clinical implementation of CTCs and ctDNA as diagnostic markers is severely limited by variability in their detection methods. With interference from background biological materials, the use of standardized and highly sensitive methods is crucial for their isolation from biofluids [30]. Indeed, CTCs are often present in low abundance among erythrocytes and leukocytes in blood samples [31], while ctDNA must be distinguished from large amounts of cfDNA secreted by non-malignant cells [32]. CTCs are commonly isolated based on their physical properties or cell surface markers [33]. Microfluidic devices like Parsortix® and ClearCell® FX1 allow CTCs to be isolated based on their size or density [34], [35], while techniques like immunomagnetic separation and flow cytometry can identify CTCs based on the expression of membrane proteins [36]. Although there is currently no clinical standard for the detection of CTCs in HNC, antibody-conjugated nanoparticles that target epithelial cell adhesion molecules have been approved for use in other malignancies, including breast, prostate, and colorectal cancers [37]. Conversely, multiple methods are available to detect ctDNA, including digital PCR (dPCR), digital droplet PCR (ddPCR), next-generation sequencing (NGS), and BEAMing (Beads, Emulsification, Amplification, and Magnetics) [38]. Although each of these methods are capable of detecting HPV ctDNA, their sensitivity can vary, with ddPCR outperforming NGS [39]. Furthermore, their accuracy in HNC might differ based on the biofluid tested and the tumor site. In HNC, HPV ctDNA detection was higher in plasma (86%) than saliva (40%) [40]. Moreover, when stratifying by tumor site rather than HPV status, salivary ctDNA detection rates are higher than plasma in oral cancer, while the opposite was seen in OPC [40]. Considering these differences, the most favorable approach appears to combine the analysis of blood and saliva, optimizing the sensitivity of HPV ctDNA detection [40], [41]. However, this may not be feasible for widespread clinical implementation, as institutions must also balance sensitivity and specificity, with factors such as patient burden, cost, and resource availability. For this reason, further investigations to optimize the accuracy of HPV ctDNA detection between biological samples and cancer sites are needed for the standardization and implementation of liquid biopsies as an auxiliary diagnostic method in HPV-positive HNC.

3.4.2 Liquid biopsies as prognostic predictors

In addition to their use in diagnostics, the quantification of HPV ctDNA has shown promise in predicting the prognosis of HPV-positive HNC patients. Research has shown associations between

the load of HPV ctDNA detected in blood and characteristics of disease, such as tumor size, cancer stage, and nodal involvement (**Table 2**), which can negatively influence patient outcomes [42], [43], [44]. A study by Hilke *et al.* corroborated that HPV ctDNA levels correlated with tumor burden and survival in HPV-positive OPC [16]. Furthermore, the presence of HPV ctDNA may also be indicative of loco-regional and distant metastasis in HPV-positive HNC patients [42], [45]. As these factors can greatly impact treatment response and patient outcomes, plasma ctDNA may be a useful tool to predict patient response and direct treatment regimens in HPV-related HNC.

In recent years, studies have tried to establish a relationship between serum HPV ctDNA, both pre-treatment and post-treatment, and patient prognosis (**Table 2**). A study by Dahlstrom *et al.* showed that patients with detectable HPV ctDNA before treatment had distinct clinical and molecular characteristics compared to patients with undetectable HPV ctDNA [43]. Additional studies demonstrated that lower pre-treatment HPV ctDNA levels followed by an increase in HPV ctDNA after commencing treatment, as well as a decrease in variant allele frequency, which provides insight on tumor heterogeneity, following the start of treatment predicted improved progression-free survival (PFS) and overall survival (OS) [44], [46]. Post-treatment ctDNA detection, on the other hand, has been extensively associated with poor prognoses, with patients exhibiting lower PFS, recurrence-free survival (RFS), and OS across multiple studies [47], [48].

Studies have also assessed the relationship between HPV ctDNA levels in biofluids and treatment response in HPV-positive HNC patients (**Table 2**). Prior to treatment, high concentrations of HPV ctDNA are often found in HNC patient biofluids, while levels are undetectable post-treatment [17], [26], [27], [42]. However, the association between HPV ctDNA load and tumor response varies in different biofluids [49]. A study by Hanna *et al.* failed to find a link between HPV ctDNA concentrations and treatment outcomes in saliva, while these factors were strongly correlated in plasma [49]. As therapies for HPV-positive HNC patients are exhibiting a shift towards less intense strategies, research on the predictive value of HPV ctDNA with these treatments is needed for appropriate treatment allocation.

Table 2. Studies examining the relationship between HPV ctDNA and treatment outcomes or patient prognoses.

Reference	Sample Size	Biopsy Type	Key Findings
[16]	20	Blood	• A time and dosage dependent decline of HPV ctDNA levels in the plasma corresponds with the primary success of the curative treatment • ctDNA is correlated with gross tumor volume before treatment

			• The tumor allele fraction in the plasma is negatively associated with the course of treatment
[17]	103	Blood	• Pre-treatment HPV ctDNA levels correlate with disease burden, tumor HPV copy number, and HPV integration status • 19 out of the 67 patients studied had a favourable ctDNA clearance profile during the treatment • Out of these 19 patients, none had persistent disease after treatment
[26]	235	Blood and saliva	• ctDNA detection was significantly higher prior to treatment than after treatment • All patients positive for HPV ctDNA before treatment showed significant reductions post-treatment • The presence of ctDNA is strongly correlated with treatment response and tumor progression in HPV-positive HNC
[27]	59	Blood and saliva	• The presence of HPV ctDNA correlates with treatment response • There are shifts in ctDNA fragment length following treatment • Patients showed major reductions in ctDNA post-treatment as compared to pre-treatment
[42]	171	Blood	• Plasma ctDNA was significantly elevated in patients with oral squamous cell carcinoma (OSCC) versus the controls • Increased plasma ctDNA levels correlated with larger tumor size, lymph node metastasis, and late stage • Higher pre-treatment plasma ctDNA levels correlated with poorer prognosis of OSCC patients
[43]	262	Blood	• Serum HPV ctDNA is associated with nodal category and overall cancer stage • Patients with detectable pre-treatment HPV ctDNA have better progression-free survival than patients who do not have detectable pre-treatment HPV ctDNA
[44]	34	Blood	• Low pre-treatment HPV ctDNA and an early increase in HPV ctDNA above baseline at week two of chemoradiation therapy were strongly associated with superior freedom from progression • Pre-treatment ctDNA values significantly correlate with nodal and metabolic tumor volume • ctDNA concentration during weeks four and seven of chemoradiation therapy was not significantly predictive of disease progression
[45]	22	Tissue and blood	• Total tumor burden strongly correlated with HPV DNA levels in the serum • Total tumor burden and median plasma HPV ctDNA levels pre-treatment negatively correlate with survival • Increasing HPV DNA levels in the plasma predict more distant anatomical tumor locations (metastasis)
[46]	53	Blood	• A change in ctDNA after one cycle of treatment is predictive of survival • A change in HPV ctDNA variant allele frequency after one cycle of treatment was predictive of PFS and OS • A decrease in variant allele frequency is linked with longer OS
[47]	295	Blood	• Detection of ctDNA at any time post-treatment is highly prognostic of poor outcomes • ctDNA status and clinical stage of disease are independently associated with patient outcomes
[48]	66	Blood	• The patients that tested positive for ctDNA before treatment had more advanced disease than those who did not

			• 83% of all deaths that occurred in the cohort had detectable ctDNA above baseline • The kinetics of pre- and post-treatment ctDNA correlated with treatment success or failure
[49]	21	Blood and saliva	• The increase and decrease in salivary HPV ctDNA levels predicted treatment response and failure in all patients with persistent locoregional disease • In paired cfDNA samples, high plasma concentration of HPV ctDNA was linked to poor treatment outcome, whereas high saliva concentration of HPV ctDNA was not
[50]	70	Blood	• Reduction of ctDNA levels below 57.9% of the baseline value at week two after treatment initiation was significantly predictive of treatment response
[51]	1	Saliva	• The HPV ctDNA load in the saliva exponentially increased in the 36-month follow-up period after diagnosis • After a bilateral tonsillectomy, salivary HPV ctDNA load was undetectable
[52]	7	Blood	• During the course of treatment, ctDNA levels declined and quickly became undetectable following tumor eradication • ctDNA levels rose upon initiation of radiation following scheduled treatment breaks
[53]	10	Blood	• Among the patients who received chemoradiation, 4 out of 7 cleared ctDNA in less than 40 days. These patients remained in remission during the follow-up period • 2 out of seven patients had persistent ctDNA after treatment. These patients became refractory to treatment

Abbreviations: HPV, human papilloma virus; ctDNA, circulating tumor DNA; cfDNA, cell-free DNA; HPV-positive, human papilloma virus-positive; HNC, head and neck cancer; OSCC, oral squamous cell carcinoma.

3.4.3 The use of liquid biopsies to monitor disease progression and recurrence

Studies have aimed to determine if HPV ctDNA detection via liquid biopsies could be used to predict cancer recurrence in HPV-positive HNC (**Table 3**). Indeed, HPV ctDNA detection in blood and saliva has been shown to predict disease progression, MRD, disease recurrence, and locoregional disease with a high sensitivity, and moderate to high specificity [26], [41], [54], [55], [56], [48], [57], [58], [59]. The presence of HPV ctDNA following treatment appears to be indicative of disease progression, locoregional recurrence, or metastasis in HPV-positive HNC [59], [60]. Many studies have reported residual tumors and HNC recurrence in patients with detectable HPV ctDNA in the bloodstream post-chemotherapy, -radiation, or -chemoradiation therapy (**Table 3**; [41], [54], [55], [61], [56], [57], [58]). Likewise, patients with ctDNA levels below baseline post-treatment have not been found to exhibit recurrences, while a detectable ctDNA viral load in blood is seen in patients with metastasis (**Table 3**; [26], [59], [60]). This is further supported by two clinical studies, which showed that HPV ctDNA was detected post-

treatment in patients that later exhibited disease recurrence [54], [56], as well as a prospective trial by Chera *et al.* which reported that recurrence did not occur in patients with undetectable HPV ctDNA following curative intent treatment [55]. Based on current literature, the quantification of ctDNA levels in the bloodstream post-treatment is a promising technique for predicting cancer recurrence in HPV-positive HNC patients.

Table 3. Studies investigating ctDNA detection in biofluids as a predictor of cancer recurrence.

Reference	Sample Size	Biopsy Type	Key Findings
[26]	235	Blood and saliva	• All participants with persistent ctDNA after treatment, except one, had residual tumor(s) and cancer recurrence • Patients with ctDNA above baseline after treatment showed evidence of disease • ctDNA detection occurred up to 18 months before clinical diagnosis
[41]	93	Blood and saliva	• Positive post-treatment salivary HPV status is linked with a higher risk of recurrence • The overall survival was reduced in patients that had a positive HPV status in the saliva post-treatment
[48]	66	Blood	• All cancer recurrences and 83% of reported deaths occurred in patients with HPV ctDNA at baseline
[54]	20	Blood	• ctDNA is a more sensitive predictor of disease recurrence than traditional imaging • ctDNA was detected in 5 of the 7 plasma samples of recurrent cases but zero cases of the recurrent-free plasma samples • There is a significant difference in post-treatment RFS time between groups with and without detected ctDNA post-treatment
[55]	115	Blood	• Detection of HPV ctDNA in two consecutive plasma samples post-treatment has a high positive predictive value and negative predictive value for identifying disease recurrence • ctDNA detection can be used to facilitate earlier initiation of salvage therapy • 28 patients had detectable HPV ctDNA during post-treatment surveillance and 15 of these patients developed biopsy proven recurrence • 16 patients had two consecutive positive HPV ctDNA blood tests and of these patients, 15 developed biopsy proven recurrence
[56]	30	Blood	• A lack of ctDNA clearance post-treatment is strongly correlated with disease recurrence • Patients with no ctDNA clearance were more likely to experience disease recurrence compared to patients with complete or partial ctDNA clearance post-treatment
[57]	N/A	Blood	• The effect of HPV ctDNA presence on disease recurrence has a hazard ratio of 7.97 • Post-treatment measurements of HPV ctDNA are more effective at predicting disease recurrence than baseline assessments
[58]	17	Tissue and blood	• Baseline ctDNA was detected in all 17 patients prior to treatment • A portion of these patients experienced disease recurrence post-treatment

			• All of the patients that experienced recurrence had detectable ctDNA 108 to 253 days before clinical diagnosis
[59]	39	Tissue and blood	• ctDNA was detected with a higher probability in metastatic recurrent cancers (70%) in comparison to locoregional recurrent disease (30%)
[61]	204	Blood	• Many patients with detectable HPV ctDNA pre-op have detectable HPV ctDNA post-op • RFS at 18 months post-op was 83% for patients with detectable HPV ctDNA and 100% for patients with undetectable HPV ctDNA

Abbreviations: HPV, Human papilloma virus; ctDNA, circulating tumor DNA; pre-op, pre-operative; post-op, post-operative.

3.4.4 Assessment of the immune microenvironment via liquid biopsy

CTCs express immune checkpoint proteins on their cell surface, as well as biomarkers which are indicative of tumor origin (e.g., EpCAM, cytokeratins, and stem cell-like markers) [62], [63]. PD-L1 (programmed death-ligand 1), a common immunosuppressive antigen, is found on the tumor cells, macrophages, and dendritic cells of cancer patients [64]. When PD-L1 binds to PD-1, immunosuppressive signals decrease T cell proliferation, thereby promoting tumor immune evasion and contributing to metastasis and poor patient prognosis [65], [66], [67]. A systematic search was conducted to determine whether liquid biopsies could be leveraged to assess the immune microenvironments of HNC patients. Following the search protocol and screening strategy (**Supplemental Material**), 304 manuscripts were identified. After the exclusion of nine duplicate studies, the reviewers excluded 253 articles based on their titles and abstracts. An additional 46 articles were excluded based on a full-text assessment (**Figure 8)**. Of these, one article was excluded based on the language (German).

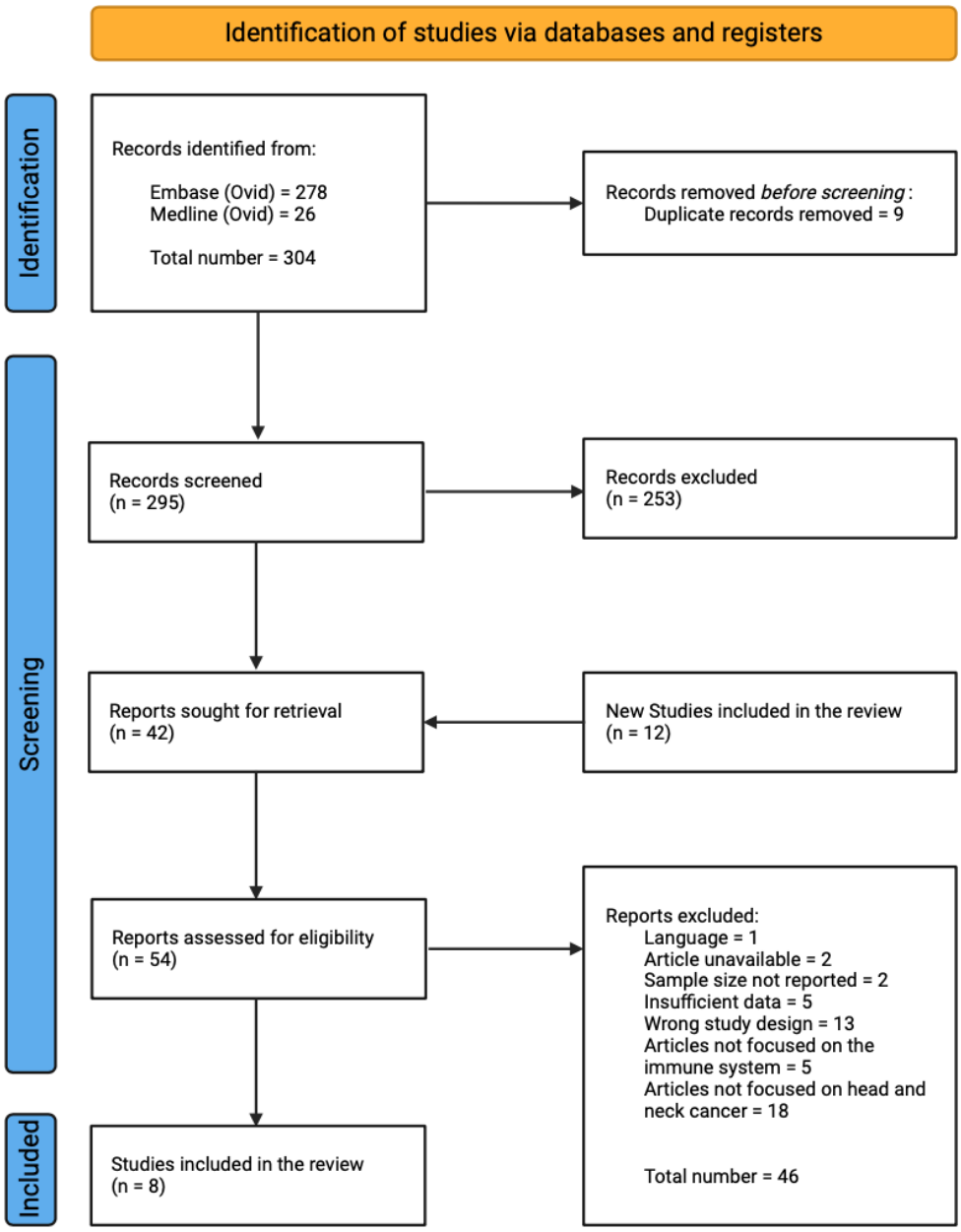

Figure 8. PRISMA flow diagram. This figure demonstrates the workflow used in the systematic review portion of this article. The stepwise process included first identification, multiple rounds of screening, and final approval of the studies that were used for analysis. The exclusion criteria used in this systematic review are listed in the screening section of the figure. Figure created using Biorender.com.

The studies included in this review were published between 2017 and 2024 and involved 814 HNC patients from three different countries (**Table S1**). Most studies were based on prospective cohorts (n = 6). The country that most characterized the immune microenvironment of HNC patients via liquid biopsy was Greece (n = 3). The majority of the research in the field centered around the clinical and prognostic impact of PD-L1-positive CTCs (n = 5). No studies

using the liquid biopsy analytes ctDNA and cfDNA were identified via the search strategy. Although data is limited, liquid biopsies have been utilized to characterize the immune systems of HNC patients. Four studies have demonstrated that the presence of PD-L1+ CTCs was significantly associated with worse patient outcomes [68], [69], [70], [71]. Patients who expressed PD-L1 on their CTCs in high quantities post-curative treatment had significantly shorter PFS and OS [71]. Likewise, the absence of PD-L1 overexpression post-curative treatment was linked with a complete response [71]. Interestingly, the expression of PD-L2 (programmed death-ligand 2) correlated with PD-L1 expression and was a significant predictor of PFS in HNC patients who received immunotherapy (pembrolizumab) [69]. Chikamatsu et al. demonstrated that there is a discordance between PD-L1 expression in the tumor tissue and in CTCs [69]. This highlights the potential of liquid biopsies as auxiliary tools in the clinical setting. Additional studies have demonstrated that surrogate biomarkers and chemokines could be used to assess the efficacy of immunotherapy and chemoradiation therapy [72], [73], [74]. For example, HNC patients with increased NANOG expression post-treatment with nivolumab showed superior disease control [72]. Furthermore, researchers have demonstrated that HNC patients with decreased levels of both PD-L1 and IDO1 (indoleamine 2,3-dioxygenase 1) mRNA post-treatment with chemoradiation therapy experienced superior OS and PFS [75]. On the contrary, HNC patients with MET-positive CTCs had significantly shorter OS in comparison to their MET-negative counterpart [72]. The risk of bias of the studies included in the systematic review was assessed and found to be minimal (**Figure S1**).

3.5 DISCUSSION

Liquid biopsies have shown promise in HPV-positive HNC, particularly through the detection of HPV ctDNA. Indeed, levels of HPV ctDNA in the plasma and saliva have been found to vary according to treatment response, with reductions seen following successful treatment [26], [27], [16], [49], [52]. This marker has also been associated with reduced survival [45], larger tumor volume, lymph node metastasis, and late clinical stages in HPV-positive OPC [42]. Similarly, strong links between HPV ctDNA and HNC disease recurrence have been revealed [26], [54], [55], highlighting its potential use in monitoring cancer progression and treatment responses. However, the clinical implementation of liquid biopsies is still cautioned in HNC, as they are severely limited by variability in the detection of ctDNA [76]. Furthermore, additional evidence is needed about

the predictive reliability of this technology [76]. Due to conflicting results between biofluid types, particularly saliva and blood which have shown different associations with patient outcomes in HPV-positive HNC [49], distinct biological fluids may require tailored methodologies to optimize the sensitivity and accuracy of their detection. Although sampling various fluid types could enhance both sensitivity and screening efficacy [77], the feasibility of this strategy may prove to be another challenge and must be considered when attempting to integrate multifluid sampling into routine clinical practice. However, to ensure its successful implementation, the cost of liquid biopsies for the diagnosis, monitoring, and management of HNC must also be elucidated. In HNC, ctDNA detection methods have been found to have lower costs [80], though they are time consuming and require laboratory expertise. Differences in ctDNA detection techniques, as well as diverse analysis and interpretation methods can lead to potential difficulties with reproducibility and inter-laboratory variability [76]. However, this concern is mitigated in the context of HPV-positive HNC, as this subtype is characterized by distinct viral biomarkers that can be consistently detected with relatively high specificity and sensitivity [49]. Nevertheless, further research is needed to address the limitations of ctDNA detection before liquid biopsies can be clinically implemented in HNC.

The immune system plays a critical role in the development, progression, and spread of cancer [81]. The systematic review conducted in this study demonstrated that the isolation and quantification of immunomodulatory proteins (e.g., PD-L1, TLR4, IRF-1, pSTAT3, and B7-H3) on the surface of CTCs via liquid biopsy can be used to predict patient prognosis and treatment outcomes [82], [83]. Pre-treatment levels of PD-L1+ CTCs are linked with poor patient prognosis, shortened PFS, and shortened OS in many cancer types, including HNC [84], [85], [68], [70]. However, PD-L1+ patients often experience a clinical benefit when treated with immunotherapeutic drugs [86], [87]. The administration of pembrolizumab and nivolumab has been recently approved for the treatment of recurrent and/or metastatic HNC [88]. Therefore, the analysis of PD-L1 expression on CTCs, as well as the analysis of circulating levels of tumoral DNA via liquid biopsy has the potential to improve the management of HNC patients in the clinic. However, this is a relatively novel field of research, with all articles in the systematic review being published within the last decade. A risk of bias assessment was conducted and the majority of the concerns stem from limited data in the field and failing to mention possible confounding factors.

Liquid biopsies which focus on immune system characterization are paving the way for personalized and effective cancer care. One such innovation is the EDIM-TKTL1/Apo10 Blood Test which isolates activated monocytes to exploit the innate immune system [89]. This technology can detect TKTL1, a marker associated with anaerobic glycolysis and metastasis, thereby allowing clinicians to monitor treatment resistance and patient outcomes [89]. In addition, a comprehensive liquid biopsy is currently under development which aims to simultaneously detect B cell malignancies and the presence of CAR-T cells in the peripheral blood of cancer patients to improve risk management and patient monitoring [90]. With extensive research and validation, liquid biopsy technologies analyzing immune cells and immune biomarkers may eventually be approved for widespread clinical implementation.

Currently, tissue biopsies remain the gold standard for cancer diagnosis, staging, and grading, as they are clinically validated and provide histological evaluations of tumors, as well as information about their spatial heterogeneity [91]. However, these techniques are invasive and unable to monitor cancer progression in real-time [92]. Liquid biopsies offer solutions to many of these issues, though it is crucial to emphasize the importance of their thorough validation and standardization [92]. To address these necessities, research dedicated towards refining of existing techniques is emphasized to promote rapid advancements in liquid biopsy technologies. While liquid biopsies may eventually become a tool in the comprehensive management of HNC patients, further research is needed to address the limitations of circulating alterations (e.g., CTCs and ctDNAs) before they can be clinically implemented.

3.6 CONCLUSION

HPV ctDNA have shown promise in diagnosis, prognostic prediction, and disease monitoring in HNC. However, their integration into routine clinical practice is challenging due to variation in their sensitivity and specificity. The biological processes regulating the dissemination of CTCs and ctDNA shedding from primary and metastatic tumors must be investigated further to optimize the detection and use of these biomarkers. Furthermore, the immune microenvironment is highly complex and plays a role in the overall survival of cancer patients. Therefore, analysis of the immune microenvironment and immunomodulatory proteins via liquid biopsy may provide a method to predict treatment responses and patient outcomes, however, further research is required.

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CHAPTER 4 – DISCUSSION

4.1 The Immune Microenvironment Plays a Role in Treatment Response and Survival

The immune system plays a critical role in cancer development and progression [206]. The four studies included in the systematic review demonstrated that the presence of PD-L1+ CTCs was linked with poor patient prognosis and decreased survival in HNC [207], [208], [209], [210]. However, PD-L1 positivity was a predictor of improved outcomes in patients undergoing immunotherapy [211], [212]. Furthermore, the difference between PD-L1 expression in the tumor tissue and the circulation hints at the potential applications of liquid biopsies in oncology [209].

The systematic review also demonstrated that the TME could be leveraged to improve the management of HNC patients. Clinicians currently exploit the immune system using immunotherapy as a means of effectively treating many types of cancer [213]. Based on the data presented in the manuscript, circulating immune biomarkers can be isolated and quantified via liquid biopsy as a means of predicting patient prognosis and treatment outcomes [207], [208], [209], [210]. However, the use of this technology to assess the immune status of cancer patients is relatively novel and, therefore, requires extensive experimental validation before being translated into the clinical setting.

Researchers are currently trying to develop new liquid biopsy technologies, such as the epitope detection in monocytes – transketolase-like protein 1/apo10 blood test, which aim to directly characterize the immune systems of cancer patients [214]. The use of liquid biopsies to analyze ctDNA levels as well as components of the immune system may offer a well-rounded tool to improve the management of HNC patients in the clinic. Advancements in this sector could propel clinicians into an era of precision oncology and subsequently improve patient survival.

4.2 The Use of Liquid Biopsy and Translational Research in Oncology

Traditional biopsies are performed to obtain tissue samples from patients that will then be examined by medical pathologists [215]. Examples of traditional biopsy techniques include fine-needle aspiration, excisional biopsy, bone marrow biopsy, and punch biopsy [215]. These procedures are usually invasive, can be painful, and are not repeatable [216]. Liquid biopsies, on

the other hand, are minimally invasive and can be performed multiple times [217]. Furthermore, they can leverage a number of bodily fluids to assess circulating analytes of interest [217].

The manuscript in Chapter 3 aimed to investigate whether liquid biopsies have applications in HPV-related HNC. Multiple studies demonstrated that the level of HPV ctDNA in the blood strongly correlated with response to treatment [218], [219], [220]. Likewise, studies have shown that the level of ctDNA decreases in a time and dosage-dependent manner following curative treatment [221]. Furthermore, multiple studies demonstrated that the detection of HPV ctDNA in the blood at any time post-treatment was linked with disease recurrence and poor patient prognosis [222], [223], [224]. Lastly, there is documented evidence that HPV ctDNA can be detected in the bloodstream prior to a diagnosis via traditional techniques [225].

Unfortunately, liquid biopsies have inherent limitations that are hindering their widespread implementation in the clinic. The main limitations are that they are not standardized, and their specificity must be optimized [226]. With a relatively large false positivity rate for several cancers, this relatively novel technique has the potential to increase cancer anxiety in patients [227], [228]. By increasing the specificity and sensitivity, we could lower the false positive rate and the subsequent anxiety that comes along with a misdiagnosis [227], [228]. However, specificity is not an issue for HPV-related HNC since viral DNA can be used as a highly specific liquid biomarker [229], [230]. To corroborate this claim, a study performed by Siravegna *et al.* demonstrated that the specificity of HPV ctDNA detection via digital droplet PCR is higher than standard diagnostic testing with p16 immunohistochemistry [231]. In addition, the sampling of multiple biofluids, such as the combination of blood and saliva, has been shown to further improve the sensitivity of HPV ctDNA detection [232]. In conclusion, liquid biopsy is a less aggressive medical test that can be used to acquire cancer-related information from body fluids. This technology could be used in the future as a complementary exam in HPV-related HNC.

CHAPTER 5 – CONCLUSIONS AND FUTURE DIRECTION

5.1 General Conclusions

This thesis presents a critical literature review of the strengths and weaknesses of liquid biopsies in the context of HPV-related HNC. Liquid biopsies have promising applications in cancer diagnosis, predicting patient prognosis, evaluating treatment response and resistance, and assessing minimal residual disease. Furthermore, the benefits of this technology include biopsy repeatability and minimal invasiveness.

The four studies included in the systematic review demonstrated that dynamic information about the immune microenvironment can be characterized via circulating analytes. The isolation and quantification of PD-L1+ CTCs via liquid biopsy may allow clinicians to better predict the prognosis and treatment outcomes of cancer patients, including those with HNC. Although liquid biopsies which harness the immune system show potential, extensive validation is required before this technology becomes widespread.

In conclusion, liquid biopsies have the potential to improve the diagnosis and management of HNC by providing a non-invasive, real-time, and comprehensive approach to tumor profiling. However, their clinical utility is currently limited by technical, interpretative, and logistical challenges. Continued research and development, along with rigorous clinical validation and standardization, is essential to overcome these limitations.

5.2 Future Directions

Although the clinical applications of liquid biopsies are vast, this technology has not yet been validated for clinical use in relation to HNC. The manuscript used in this thesis provided a critical review of how this technology could revolutionize the current standard of care for HNC. However, the clinical implementation of this relatively novel laboratory technique is hindered by a lack of standardization and low analyte specificity. Future studies should compare the efficacy of common analysis methods (ddPCR, next-generation sequencing, BEAMing, etc.) to develop a standardized analytical protocol. In addition, a cost analysis study should be performed comparing standard diagnostic methods used in the clinic to liquid biopsies. If the latter proves to be a more cost-effective cancer management strategy, its adoption could pave the way for greater global

accessibility to cancer care. Furthermore, future studies should investigate whether HPV ctDNA levels in the circulation are linked with patient immune status. Translating research findings into clinical practice is essential for improving the care of cancer patients. Therefore, the findings in this manuscript highlight promising new avenues for research and clinical trials. However, the incorporation of liquid biopsies into clinical practice will require further validation and rigorous testing to ensure their accuracy and reliability.

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SUPPLEMENTAL MATERIAL

Search Strategy

Embase Classic+Embase <1947 to 2024 Week 24> *

1 (cancer* or carcinoma* or neoplasm* or malignan* or metastas*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
2 "squamous cell carcinoma of head and neck"/ or oropharyngeal neoplasms/ or tonsillar neoplasms/
3 1 or 2
4 Liquid Biopsy/
5 circulating tumor cell/
6 3 and 4
7 (immun* or immune system).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]
8 5 and 6
9 7 and 8

* Equivalent key words and mesh terms were used to search the Medline (Ovid) database.

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 37
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Page 38
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Page 40
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 41
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Page 42
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 41
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Supplemental Material
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Page 43
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Page 43
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Page 43
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Page 43
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 43
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Page 43
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Page 41
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Page 43

Section and Topic	Item #	Checklist item	Location where item is reported
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Page 43
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Page 43
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	N/A (no methods were used to assess heterogeneity of results)
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Page 43
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Page 43
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	N/A (no statistical methods were employed)
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 50-51
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Page 50
Study characteristics	17	Cite each included study and present its characteristics.	Page 51
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Supplemental material (Figures S1)
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Page 52
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Page 50-52
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	N/A (no statistical methods were employed)
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	N/A (no statistical methods were employed)
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A (no statistical methods were employed)
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Page 52

Section and Topic	Item #	Checklist item	Location where item is reported
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	N/A (no statistical methods were employed)
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Page 53
	23b	Discuss any limitations of the evidence included in the review.	Page 53
	23c	Discuss any limitations of the review processes used.	Page 53
	23d	Discuss implications of the results for practice, policy, and future research.	Page 53
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Page 41
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Page 41
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A (no amendments to the protocol were made)
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Page 55
Competing interests	26	Declare any competing interests of review authors.	Page 55
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Page 55

PRISMA 2020 Checklist. Available at: https://www.prisma-statement.org/prisma-2020.

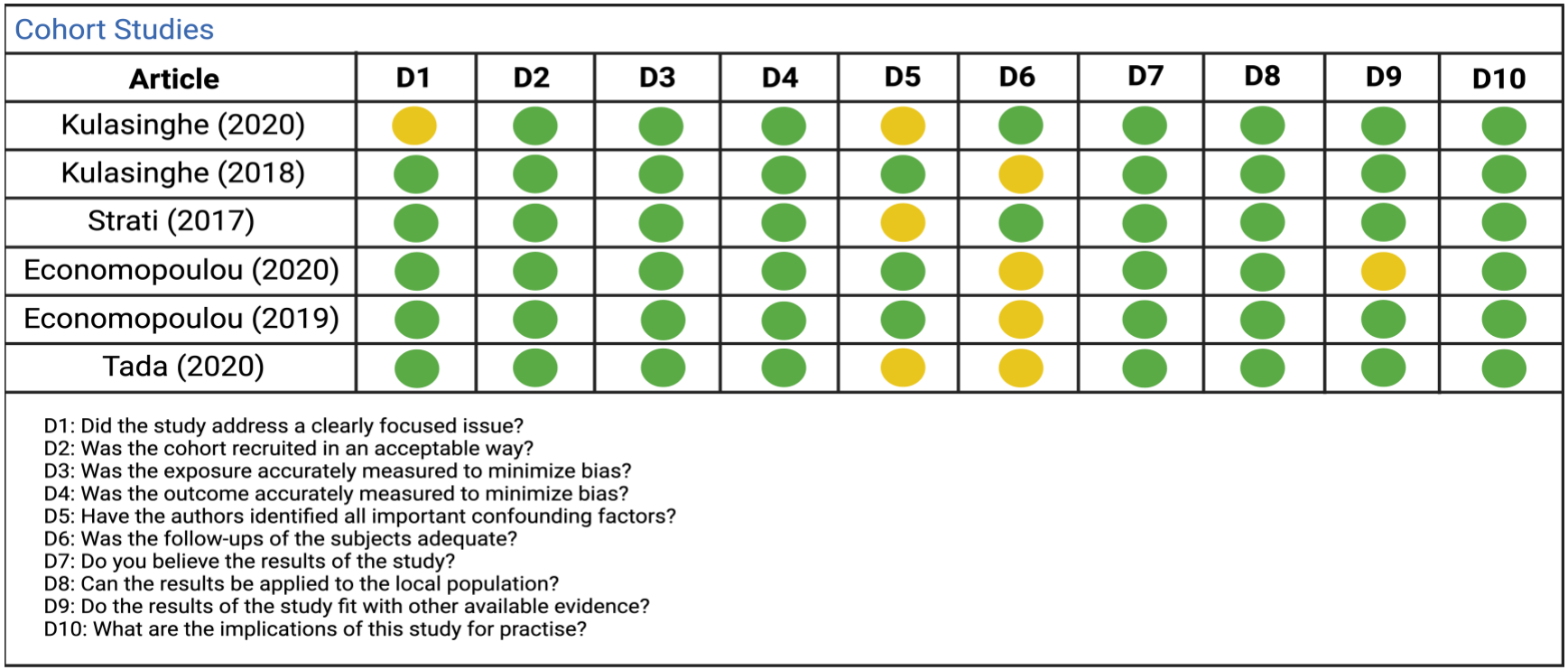

Figure S1. Risk of bias assessment for articles in the systematic review. Eight studies were identified via the database search strategy. However, two studies were excluded from the risk of bias assessment due to incompatible study type. *Critical Appraisal Skills Programme (2023). CASP Cohort Study Checklist. [online] Available at: https://casp-uk.net.*

Table S1. Characteristics of the studies included in the systematic review.

Author (year)	Country	Study Design	Sample Size	Cancer Type	Liquid Biopsy Analyte	Evaluation of Patient Survival	Evaluation of Treatment Outcome
Kulasinghe (2020)	Australia	Prospective cohort study	350	Head and neck cancer	CTC	Yes (PFS)	No
Chikamatsu (2019)	Japan	Prospective observational study	30	Head and neck cancer	CTC	Yes (PFS)	Yes
Kulasinghe (2018)	Australia	Prospective cohort study	23	Head and neck cancer	CTC	Yes (PFS)	No
Strati (2017)	Greece	Prospective cohort study	113	Head and neck cancer	CTC	Yes (OS and PFS)	Yes
Tada (2021)	Japan	Observational or cross-sectional study	42	Head and neck cancer	CTC	Yes (OS)	No
Economopoulou (2020)	Greece	Prospective cohort study	113	Head and neck cancer	CTC	Yes (OS and PFS)	Yes
Tada (2020)	Japan	Prospective cohort study	30	Head and neck cancer	CTC	Yes (OS)	Yes
Economopoulou (2019)	Greece	Prospective cohort study	113	Head and neck cancer	CTC	Yes (OS and PFS)	Yes

Abbreviations: OS, overall survival; PFS, progression-free survival; CTC, circulating tumor cell.