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# Emerging Proximal Liquid Biopsy Approaches for Detecting Residual Disease and Predicting Recurrence in Head and Neck Cancer: A Review and Proposal of Novel Liquid Staging

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## ABSTRACT

**Background:** Head and neck squamous cell carcinoma remains challenging due to high recurrence rates and poor survival outcomes. Developing precise technologies for disease burden assessment, treatment response, and minimal residual disease (MRD) surveillance is crucial for improving prognosis.

**Methods:** This review explores the potential of liquid biopsy for MRD and recurrence detection. A novel liquid TNM (LiTNM) staging system is introduced, integrating biomarkers from saliva, surgical drain lymphatic fluid (SLF), and peripheral blood.

**Results:** Proximal liquid biopsies, particularly saliva and SLF, offer advantages due to their proximity to the tumor microenvironment. Saliva demonstrates high sensitivity in HPV-associated oropharyngeal cancers, while SLF holds potential in identifying early postoperative recurrence. Despite these advancements, standardization and validation remain challenges.

**Conclusions:** Liquid biopsy approaches show promise for postoperative disease monitoring, yet their clinical implementation remains in the early stages. The proposed LiTNM staging system could complement TNM staging by providing a molecular framework for risk stratification. However, rigorous prospective studies are necessary to validate its clinical utility and facilitate adoption.

## 1 | Introduction

Head and neck squamous cell carcinoma (HNSCC) represents a significant global health challenge, accounting for approximately 4% of all cancers worldwide [1]. In the United States, an estimated 58,450 new cases of oral cavity and pharynx cancers are projected in 2024 [2]. Despite advances in treatment modalities, HNSCC remains difficult to manage due to its high rates of recurrence and limited improvement in survival outcomes [3]. Recurrent and metastatic HNSCC are particularly associated with poor prognosis, with a median survival time ranging from 6 to 15 months [4, 5]. Thus, earlier detection of recurrence

is critical, as timely interventions such as salvage surgery or re-irradiation can significantly improve survival [6].

Despite ongoing research into improved surveillance methods, current clinical practice for detecting residual disease and subsequent recurrence still relies heavily on traditional approaches such as imaging, biopsies, postoperative surgical pathology, and clinical evaluations. While these methods remain integral to patient management, they have significant limitations. These methods can be invasive and inconsistent in detecting small residual tumors or early relapses, particularly for tumors in less accessible anatomic locations [7–9]. This underscores the need for more sensitive diagnostic tools to identify postoperative residual disease and predict recurrence.

Minimal residual disease (MRD) detection via liquid biopsy has emerged as a potentially valuable complementary tool to traditional diagnostic and surveillance methods in cancer care [10]. By analyzing biofluids such as blood and saliva, liquid biopsy can identify tumor-specific molecular markers, including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and extracellular nucleic acids, which collectively comprise the tumor circulome [10–12]. This approach offers a minimally invasive method for the early detection of MRD and the assessment of recurrence risk, with growing evidence suggesting that liquid biopsy can identify recurrence earlier than traditional imaging techniques [13, 14]. While the use of liquid biopsy is a rapidly emerging technology across various solid tumor malignancies, like colorectal and non-small-cell lung cancer [15, 16], its application in HNSCC is still in the early stages, and clinical guidelines for its use are lacking.

In this review, we explore the evolving landscape of MRD and recurrence detection in HNSCC using plasma-based liquid biopsy and emerging proximal biofluids, particularly saliva and surgical drain fluid. We also propose a novel molecular staging framework, liquid TNM (LiTNM), that incorporates these proximal and distal biomarkers to improve the detection of residual disease, predict recurrence, and guide personalized postoperative treatment.

## 2 | Plasma-Based Liquid Biopsy

Plasma, a readily accessible biofluid from peripheral blood, has been extensively investigated for its application in HNSCC liquid biopsy. It serves as a reservoir for tumor-derived markers such as ctDNA, CTCs, and exosomes. Among these markers, ctDNA has garnered the most attention in HNSCC research. Released into the bloodstream by necrotic or dividing tumor cells, ctDNA fragments can provide a dynamic representation of the tumor's genetic landscape and biologic activity.

Advances in next-generation sequencing (NGS) and digital droplet PCR (ddPCR) have facilitated the detection of ctDNA through the analysis of genetic mutations and epigenetic modifications. For instance, Wilson et al. assessed pre-treatment plasma ctDNA from 75 patients with either recurrent or primary HNSCCs and found genetic alterations in *TP53*, a hallmark of HNSCC oncogenesis, to be the most frequent tumor-specific mutation in the ctDNA and showed that *TP53* mutant-containing ctDNA detection was significantly associated with inferior overall survival. [17] Similarly, Porter et al. utilized NGS in a cohort of 60 patients demonstrating that other canonical HNSCC mutations, such as *NOTCH1* and *PIK3CA*, could be used to detect plasma ctDNA [18]. Overall, paired tumor-plasma analysis yields varying levels of tissue mutational concordance, dependent in part on the molecular assay modality [19].

Interestingly, recent studies in HNSCC have leveraged emergent highly sensitive assays to detect evidence of MRD following curative intent treatment. One such example is the LIONESS study, which utilized RaDaR, a tumor-personalized assay capable of detecting variant allele frequencies as low as 0.0006%, to detect MRD in all patients who suffered recurrence, with lead times on medical imaging ranging from 108 to 253 days [20]. Furthermore, dynamic changes in plasma levels of ctDNA burden during post-radiotherapy cancer surveillance allowed for earlier treatment response interpretation and imminent disease progression compared to standard of care medical imaging [21–23].

In patients with human papillomavirus (HPV)-positive HNSCC, circulating tumor HPV DNA (ctHPV DNA) can be tracked after oncologic surgery alongside tumor-specific mutations [24, 25].Studies have shown that ctHPV DNA assays can detect recurrence months before imaging, with lead times ranging from 53 days up to 18 months, and exhibit high sensitivity (82%–98%) and specificity (97%–100%) [23]. Notably, ctHPV DNA alone can be used as a biomarker for MRD monitoring in patients treated with curative intent [26, 27]. While the development of ctHPV DNA assays is ongoing by academic groups, including ctHPV16 DNA assays [28], the commercial tumor tissue-modified viral HPV DNA (NavDx, Naveris Laboraties) has permitted widespread adoption in clinical settings [29, 30]. Plasma ctHPV DNA liquid biopsies have also proven useful in detecting distant relapses [31], highlighting their potential in long-term patient monitoring. Other virus-informed ctDNA assays have been used. For example, PCR-based detection of circulating tumor Epstein–Barr Virus DNA (ctEBV DNA) positivity has been associated with poorer survival outcomes [32]. Despite these promising findings, ongoing research is needed to determine whether biomarker-based surveillance improves survival compared to conventional follow-up methods.

CTCs, another key component in plasma-based liquid biopsy, are tumor cells that have detached from the primary tumor and entered the bloodstream. Elevated CTC counts have been linked to tumor progression, a higher recurrence risk, and poor prognosis [33, 34]. More recently, circulating exosomes, such as micro ribonucleic acid (miRNA), have been explored as biomarkers. Studies suggest that detecting miRNA may enable prognostication of patient outcomes, ultimately predicting locoregional recurrence in HNSCC [35]. However, research on exosomes in HNC is still in the early stages, and more work is needed to establish their clinical utility.

## 3 | Proximal Liquid Biopsy

While blood-based biomarkers have shown promise, their limitations should be considered, namely signal degradation due

to the distance from the primary tumor [36]. For instance, the amount of tumor-derived biomarkers released into the bloodstream can vary significantly, with biomarkers like ctDNA having a half-life of mere minutes to hours [37]. Furthermore, peripheral cell-free DNA (cfDNA) dilutes the concentration of ctDNA, resulting in a "needle in a haystack" search [36]. This results in challenges in identifying tumor-specific mutations or alterations, particularly when tumor-derived biomarkers are present at low levels. In addition, the variability in assay sensitivity, the lack of standardized collection and processing protocols limit their reproducibility. These challenges highlight the need for more localized accessible biofluid sources beyond the blood [36], to enhance biomarker detection.

This has led to increased interest in proximal liquid biopsy, utilizing biofluids in close proximity to the primary tumor. Fluids such as cerebrospinal fluid, pleural fluid, urine, and stool have shown promising results in detecting tumor markers in other cancers [38–41]. For example, classifying MRD using urine ctDNA multi-omics profiling significantly outperformed plasma ctDNA in predicting survival in localized bladder cancer patients [42, 43] and also uncovered a stemness phenotype that predicted inferior survival in androgen deprivation-resistant prostate cancer [44]. Similarly, pleural fluid in lung cancer has been identified as a rich source of ctDNA and tumor-associated T cells, offering greater sensitivity and specificity in predicting treatment response than peripheral blood markers [45–47]. In the context of head and neck, saliva and surgical drain fluid have emerged as potential proximal biomarker sources.

### 3.1 | Saliva as Proximal Biofluid

Saliva is gaining recognition as a non-invasive promising liquid biopsy for monitoring MRD and predicting recurrence in HNSCC [48]. It contains various molecular biomarkers that can possibly reflect the molecular landscape of tumors such as DNA, RNA, miRNAs, and exosomes [49]. These biomarkers span genetic mutations, epigenetic alterations, and molecular changes linked to cancer development and recurrence [50]. The benefits of saliva as a biomarker are similar to those of other proximal biofluids, such as its ease of accessibility and proximity to the tumor.

Studies have demonstrated that tumors shed ctDNA into saliva, providing a valuable source for disease monitoring. Salivary ctDNA has been shown to detect somatic mutations at mutant allele fractions as low as 0.015% [51–53]. Sequencing salivary ctDNA, especially HPV DNA, has revealed high specificity and varying levels of sensitivity in capturing MRD and tracking disease progression and recurrence [54, 55]. Notably, a five-year follow-up study further underscored the prognostic value of detecting high-risk HPV (HR-HPV) DNA in saliva, showing a significant correlation with improved patient survival. [52] Beyond ctDNA, advancements in detecting HR-HPV E6 and E7 oncoproteins in saliva have shown promise for enhancing disease surveillance in patients with OPSCC [56].

The integration of saliva and plasma biomarkers has further improved recurrence detection. For instance, detecting HPV16 DNA in both saliva and plasma significantly enhances early recurrence detection in HPV-positive OPSCC, underscoring the complementary potential of multiple liquid biopsy sources [57]. Some studies suggest that salivary ctDNA has improved locoregional MRD detection in HPV-related HNSCC, whereas plasma provides superior sensitivity for detecting distant MRD, providing a rationale for combining these assay types [31]. Currently, saliva-based liquid biopsies have reached clinical application, with validated tests for HPV-positive HNSCC now aiding in both screening and guiding treatment decisions [58]. However, these innovations have largely focused on HPV-associated tumors in the oral cavity and oropharynx. Challenges persist in extending these approaches to HPV-negative HNSCC, where high mortality rates underscore the urgent need for more effective diagnostic tools [23].

Other than HPV-specific markers, preliminary studies have shown that saliva offers valuable information on genetic mutations and epigenetic alterations in HNSCC. Mutations in genes such as TP53, NOTCH1, EGFR, and PIK3CA have been identified in saliva samples, offering potential for risk stratification and disease monitoring [59–61]. Methylation markers, including p16, MGMT, DAPK, and TIMP3, have also shown promise in correlating with HNSCC risk of recurrence [62].

Salivary miRNAs, involved in gene expression regulation, have also gained attention as important biomarkers for detection and disease progression [63]. A meta-analysis highlighted specific salivary miRNAs, including miR-146a, miR-155, miR-31, miR-21, miR-10b, and miR-375, that correlate positively with the severity of OPSCC [64]. Furthermore, miR-139-5p levels, which decrease in patients with tongue squamous cell carcinoma and return to baseline after tumor resection, demonstrate their potential for monitoring treatment response and recurrence [65].

Salivary cytokines, including tumor necrosis factor-alpha (TNF-α), interleukins (IL-6, IL-8), and vascular endothelial growth factor (VEGF), have been identified in patients with HNSCC. Elevated levels of these cytokines have been correlated with tumor burden, poor treatment response, and survival outcomes [66–68]. A recent study found significantly higher TNF-α levels in the saliva of patients with OSCC compared to those with healthy controls and patients with oral leukoplakia [68]. These findings highlight the potential of salivary TNF-α as a prognostic marker of OSCC. Together, these findings emphasize the value of saliva as a proximal biofluid, providing molecular insights that could improve the detection, management, and prognosis of HNSCC. While significant progress has been made, current studies remain exploratory with small patient cohorts. Salivary biomarkers have yet to overcome challenges before achieving widespread clinical adoption. A key limitation is the lack of standardization in saliva collection, processing, and analysis. For example, patients undergoing radiation therapy often face difficulty producing sufficient saliva due to damage to the salivary glands, leading to decreased secretion [69]. Variability in the saliva collection process, including the time of collection, diet, oral hygiene, and hydration levels, adds another layer of complexity [70]. Additionally, the type of saliva collected—stimulated versus unstimulated—can result in different biomarker profiles [71]. This is especially relevant in patients undergoing radiotherapy, as stimulated saliva may show a reduction in important oral proteins involved in oral health,

such as those related to apoptosis, antibacterial functions, and acid resistance [71]. Variability in processing, including inconsistent centrifugation speeds, variations in storage conditions, and the occasional use of ribonuclease or proteinase inhibitors to preserve the integrity of salivary proteins and RNA, can also affect the sample quality and biomarker integrity [70].

There is also a lack of standardization in detection methods, with studies employing techniques such as NGS, ddPCR, enzyme-linked immunosorbent assay (ELISA), and mass spectrometry [49, 72]. Despite the growing body of literature, consensus on the most reliable biomarkers and detection techniques remains unclear. The high variability in biomarker performance across studies underscores the necessity of standardization of detection techniques, assay sensitivity improvements, and validation in large, prospective clinical trials. While saliva-based assays have demonstrated success in HPV-positive HNSCC, translating these advances to HPV-negative tumors and other aggressive subtypes is also essential.

### 3.2 | Surgical Drain Lymphatic Fluid as Proximal Biofluid

Surgical drain lymphatic fluid (SLF) has emerged as a novel liquid biopsy, leveraging its proximity to the surgical site to capture tumor-derived biomarkers. SLF is defined as the serosanguinous fluid captured in surgical drains that evacuate the neck wound bed following primary tumor excision and neck lymph node dissection. Indeed, preliminary data support the assertion that this surgical drain fluid is lymphatic in nature, as it is enriched with lymphatic-associated proteins (ANXA1, ARG1, and S100A4) [73]. The unique proximity advantage of SLF enhances the sensitivity of early postoperative MRD detection and recurrence prediction. By analyzing MRD early, SLF has the potential to guide future personalized intervention efforts, including treatment de-escalation for selected patients.

Recent evidence highlighted the utility of ctDNA in SLF for postoperative surveillance and early recurrence detection [74]. Tumor-informed ctDNA mutations, particularly in genes associated with HPV-positive OPSCC—such as those involved in the PI3K pathway, DNA repair, and chromatin stability—have been identified in SLF [74]. Notably, lymphatic fluid from surgical drains has been shown to contain significantly higher levels of ctHPV DNA than plasma in HPV-positive HNSCC patients following resection, highlighting its enhanced sensitivity in detecting tumor-derived DNA [74]. Recently, a study by Loo et al. highlighted the high sensitivity of SLF in detecting ctHPV16 DNA using ddPCR [28]. For instance, SLF showed a significantly higher detection rate of HPV16 DNA compared to plasma [28].

Interestingly, some patients who underwent adjuvant chemoradiotherapy following oncologic surgery exhibited no detectable MRD in their lymphatic fluid, suggesting that treatment de-escalation could have been a safe and effective option for these individuals [74]. In addition, patients stratified as high-risk based on postoperative MRD models exhibited substantially worse progression-free survival. [74] This highlights the potential of SLF biomarkers in predicting outcomes and guiding treatment, ultimately enhancing postoperative care.

Beyond ctDNA, SLF and other similarly harvested lymph analogs harbor clinically relevant acellular biomarkers, including matrix metalloproteinases (MMPs), cytokines, and angiogenesis-related factors. These biomarkers provide insights into tumor progression, immune modulation, and wound healing [75]. For instance, retrospective studies in head and neck cancer populations have shown that patients who eventually suffered disease recurrence had significantly lower levels of MMP-1 and MMP-3 in their postoperative lymph fluid [75–77]. Similarly, cytokines like soluble tyrosine kinase inhibitor type 1, a VEGF antagonist, have shown prognostic relevance, with lower levels associated with tumor recurrence [75]. Lastly, emerging cytometric analysis of SLF is just beginning to elucidate its unique immune cell composition. Compared to patient-matched plasma, surgical lymph has a significant enrichment of natural killer (NK) cells, and the lymph of patients who recurred has elevated levels of CD56dimCD16+ NK cells compared to patients without disease recurrence [78]. Future studies will aim to characterize the prognostic importance of both the cellular and acellular biomarkers of SLF.

By complementing traditional monitoring methods, SLF presents a promising avenue for providing sensitive and timely insights into disease progression and recurrence risk. Despite ongoing research, it is crucial to acknowledge that its clinical application remains in the early investigational stages. Current evidence is limited, with few studies, small sample sizes, and a lack of standardized methodologies. Rigorous efforts are needed to establish its clinical feasibility and validation.

Future research must focus on large-scale prospective studies to confirm its prognostic utility. Expanding the range of detected biomarkers associated with tumor progression, prognosis, and wound healing is essential for a comprehensive understanding of SLF composition. Given the dynamic physiological changes in postoperative wound healing—such as cytokine release, cellular migration, and extracellular matrix deposition—it is crucial to determine baseline biomarker levels, identify the most informative markers, and assess their reproducibility across diverse patient populations and disease stages. Additionally, further studies should determine the optimal collection process, appropriate collection timing at different postoperative stages, and proper processing and analysis techniques.

## 4 | Liquid TNM (LiTNM) Staging: A Conceptual Framework

The American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) staging system for solid tumors was first conceptualized in 1954 and is now in its 8th edition for HNSCC [79]. The tumor (T), node (N), and distant metastasis (M) classification stratifies patients into distinct prognostic groups by defined clinical or histopathologic features. For HNSCC, TNM staging is the cornerstone of disease prognostication and patient counseling, informs treatment recommendations, and permits a simple, uniform language for clinical and research communications throughout the world [80]. Since the 1950's, each new iteration of the TNM staging system has sought to incorporate contemporary insights into the clinical and biological behavior of HNSCC to improve its clinical utility [81]. The major changes from the 7th to 8th edition

of the TNM staging system were the creation of distinct categories for HPV-related versus HPV-unrelated OPSCC, incorporation of depth of invasion (DOI) into the T category for oral cavity SCC, and incorporation of extranodal extension (ENE) into the N category for HPV-unrelated HNSCC [82].

The optimal cancer staging system is highly predictive of survival outcomes and maximizes hazard consistency (i.e., intra-stage survival outcomes are similar), hazard discrimination (i.e., inter-stage survival outcomes are distinct), and balance between groups (i.e., similar number of patients in each stage) [83]. Initial evidence suggests the 8th edition TNM staging system for HNSCC has improved on these parameters overall. [79] However, current criticisms leveraged at the newest edition include the absence of ENE sub-categories (e.g., minor versus major) [84] and de-emphasis on the number of positive lymph nodes, rather than node size and laterality, in HPV-negative HNSCC [85]. Undoubtedly, the TNM Staging system for HNSCC is imperfect and is likely not sufficiently capturing features of HNSCC tumor immunobiology that define particularly aggressive cancers with poorer survival outcomes. This problem is particularly evident in early-stage patients who experience recurrence and mortality despite standard-of-care oncologic treatment.

Validation of novel staging systems for HNSCC that integrate genomic signatures of aggressive tumor biology must show practicality, cost-effectiveness, and rapid assay turnaround time to guide timely treatment decisions [86]. While numerous such signatures have been proposed for prognostication and treatment selection in both HPV-related and -unrelated HNSCC, translation to routine clinical practice has not yet been realized. Emerging liquid biopsy approaches that assay tumor DNA, RNA, and other biomarkers may revolutionize HNSCC staging systems for enhanced prognostication and treatment selection in the coming years [87].

Our laboratory is actively working to validate a novel LiTNM staging system leveraging saliva, SLF, and peripheral blood as liquid correlates to primary tumor (T), lymph nodes (N), and distant metastasis (M), as depicted in Figure 1. Our LiTNM concept utilizes sensitive detection of tumor-informed ctDNA and HPV cfDNA in saliva, SLF, and peripheral blood perioperatively to molecularly restage patients into distinct risk categories for cancer recurrence. Preliminary findings support this concept [28, 73, 74, 78], with recent work demonstrating the feasibility of a ddPCR-based HPV16 detection assay in plasma, serum, and SLF samples [28]. While ongoing studies continue, our active collection of saliva, SLF, and peripheral blood will be instrumental in establishing the clinical validity and utility of LiTNM staging.

Several distinct advantages of our approach are thus proposed. First, our LiTNM concept leverages minimally invasive

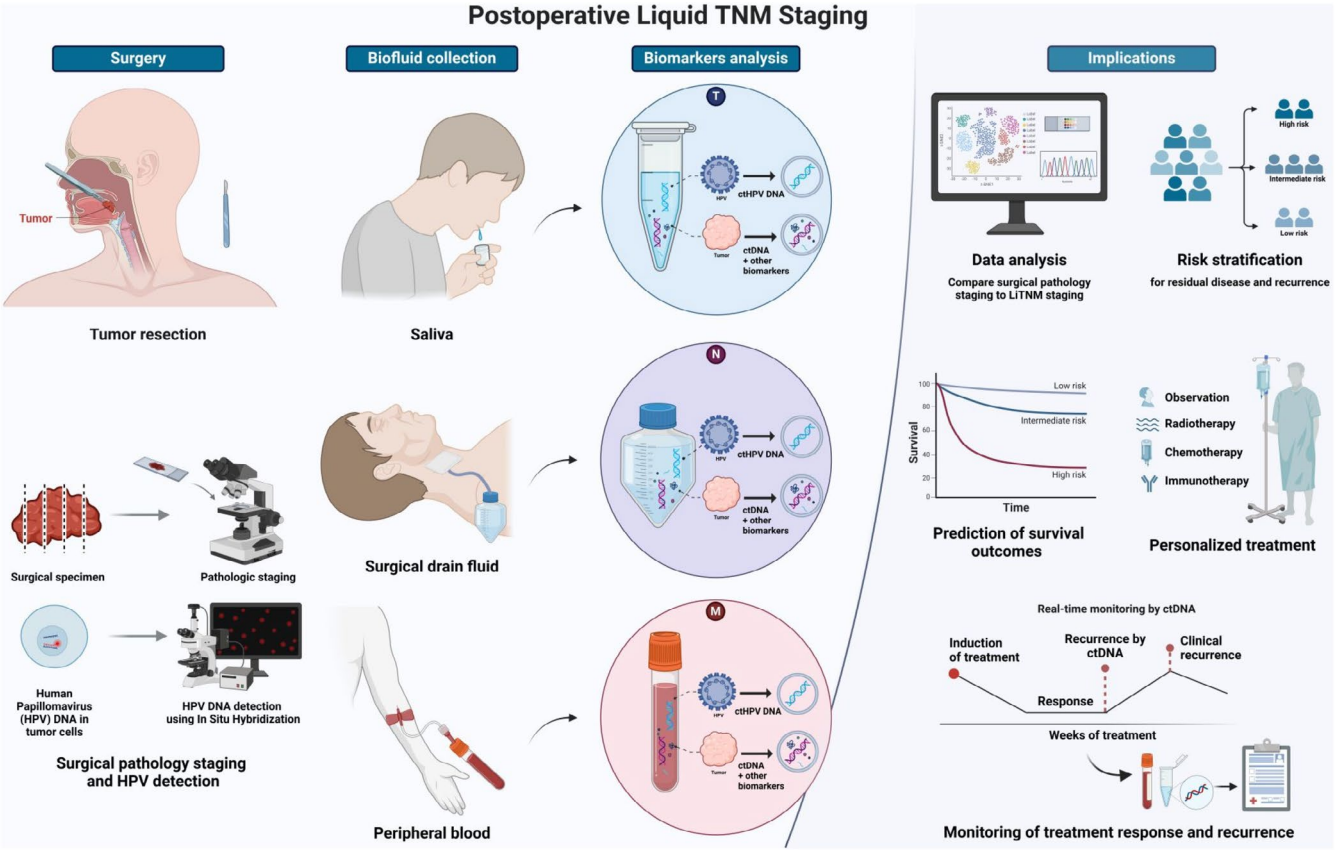


**FIGURE 1** | A novel liquid TNM (LiTNM) staging system for HNSCC integrating biomarker analysis from saliva, surgical drain fluid, and peripheral blood for detecting minimal residual disease, predicting recurrence, and guiding postoperative treatment. CtDNA, circulating tumor DNA; ctHPV DNA, circulating tumor human papillomavirus DNA; LiTNM, Liquid tumor (T), node (N), and distant metastasis (M) staging. [Color figure can be viewed at wileyonlinelibrary.com]

collection of multiple proximal biofluids representative of distinct tumor compartments. Second, our LiTNM system would utilize a high-sensitivity and high-throughput assay with rapid turnaround time capable of quantizing the stage of *remaining* post-surgery residual disease (imputed from MRD burden across saliva, lymph, and plasma) complementing the traditional pathologic TMN system which stages cancerous tissue that has already been surgically resected.

However, it is crucial to emphasize that the LiTNM staging system is a conceptual framework, and its clinical application is not yet validated. While preliminary research supports the potential of liquid biopsy for MRD detection, there is currently insufficient evidence to justify a formal revision of post-treatment staging guidelines. Extensive clinical validation through prospective studies is necessary to assess the prognostic accuracy and reproducibility across institutions of the proposed staging system.

## 5 | Conclusions and Future Directions

Emerging evidence underscores the potential of biofluids, such as saliva and surgical drain lymphatic fluid, to provide valuable insights into disease dynamics, particularly in the postoperative setting. The integration of multiple liquid biopsy approaches into clinical practice holds great promise for improving disease monitoring, enhancing the detection of residual disease, and predicting recurrence in head and neck cancer. By incorporating these insights, there is an opportunity to refine the AJCC/UICC TNM staging system, addressing its limitations and integrating the dynamic nature of tumor biology into prognostic models.

Despite these advancements, liquid biopsy research remains in an investigational stage and faces critical challenges before clinical adoption. Establishing standardized thresholds for biomarkers is crucial to ensure consistency and accuracy across studies. Additionally, optimizing assay sensitivity, reproducibility, and cost-effectiveness is imperative for broader clinical application. Successful implementation also requires streamlined workflows and rapid assay turnaround times to support timely clinical decision making.

The proposed LiTNM staging system aims to integrate tumor-specific genomic signatures for improved risk stratification; however, its implementation is constrained by the lack of robust clinical validation of liquid biopsy. While this system has the potential to guide treatment de-escalation or intensification—ultimately improving survival outcomes and potentially redefining the standard of care in HNSCC—its feasibility and impact must first be rigorously tested.

Future research must prioritize large-scale prospective clinical trials to validate individual liquid biopsies and ultimately test the LiTNM staging system's ability to detect residual disease and predict recurrence. Incorporating sequential liquid biopsy sampling at various stages of the disease will also be crucial for developing dynamic monitoring protocols that align with personalized treatment strategies. With continued research, these efforts could pave the way for a new era of precision oncology.


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### Ethics Statement

Institutional review board approval is not required for this review.

### Consent

The authors have nothing to report.

### Conflicts of Interest

Jose P. Zevallos is a founder and equity shareholder in Droplet Biosciences. The other authors declare no conflicts of interest.

### Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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