Liquid Biopsy in Head and Neck Cancer: Current Evidence and Future Perspective on Squamous Cell, Salivary Gland, Paranasal Sinus and Nasopharyngeal Cancers

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Simple Summary: Head and neck cancer is the sixth most common type of solid tumor and harbors a poor prognosis since most patients are diagnosed at an advanced stage. The study of different tumor components in the blood, saliva or other body fluids is called liquid biopsy. The introduction of novel diagnostic tools such as liquid biopsy could aid in achieving earlier diagnoses and more accurate disease monitoring during treatment. In this manuscript, the reader will find an in-depth review of the current evidence and a future perspective on the role of liquid biopsy in head and neck cancer.

Abstract: Head and neck cancer (HNC) is currently the sixth most common solid malignancy, accounting for a 50% five-year mortality rate. In the past decade, substantial improvements in understanding its molecular biology have allowed for a growing development of new biomarkers. Among these, the field of liquid biopsy has seen a sustained growth in HNC, demonstrating the feasibility to detect different liquid biomarkers such as circulating tumor DNA (ctDNA), circulating tumor cells (CTC), extracellular vesicles and microRNAs. Liquid biopsy has been studied in HPV-negative squamous cell carcinoma of the head and neck (SCCHN) but also in other subentities such as HPV-related SCCHN, EBV-positive nasopharyngeal cancer and oncogene-driven salivary gland cancers. However, future studies should be internally and externally validated, and ideally, clinical trials should incorporate the use of liquid biomarkers as endpoints in order to prospectively demonstrate their role in HNC. A thorough review of the current evidence on liquid biopsy in HNC as well as its prospects will be conducted.

Keywords: liquid biopsy; head and neck cancer; circulating tumor cells; CTC; circulating tumor DNA; ctDNA; human papillomavirus; HPV; Epstein-Barr virus; EBV; extracellular vesicles; microRNAs

1. Introduction

Head and neck cancer (HNC) is the sixth most common solid tumor and is the eighth most common cause of mortality [1]. Sixty percent of HNCs present with locally advanced disease. Unfortunately, up to 50% of these patients will relapse within the next three years, in most instances as an incurable disease [2]. In addition, HNC is one of the cancers with the highest impact on quality of life and social functioning due to the anatomic and functional areas involved [3]. Earlier disease diagnosis and identification of relapse would help to improve this poor prognosis and the functional outcomes after therapy. The field of liquid biopsy has been one of the leading research areas in oncology during the past fifteen years. While more broadly developed in breast, lung, colorectal and prostate cancers, notable milestones have been attained in other less common entities such as head and neck cancer (HNC). Being the sixth most common solid tumor and the eighth most common cause of cancer death worldwide, improvements in understanding its biology and the refinement of molecular diagnostics have paved the way for the introduction of...
liquid biopsy in HNC. A growing number of studies using different technologies, such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), micro-RNAs (miRNAs) and extracellular vesicles (EVs), have demonstrated the feasibility of applying liquid biopsy as a prognostic and/or predictive tool in patients with HNC [4]. However, the lack of internally and externally validated studies, as well as the scarcity of prospective liquid biomarker trials in HNC, limits our understanding of liquid biopsy in this disease and prevents its widespread incorporation into clinical practice [5]. On the other hand, although HNC’s complex biology and lack of frequent hotspot mutations limit its development as a predictive tool, a growing number of studies have demonstrated the predictive value of CTCs and/or ctDNA in HPV-unrelated squamous cell cancer of the head and neck (SCCHN) as well as in certain sub-entities such as human papillomavirus (HPV)-related oropharyngeal cancer (OPC), Epstein-Barr Virus (EBV) nasopharyngeal cancer (NPC) and certain salivary gland carcinomas [6–8].

The present review will summarize the current state-of-the-art of liquid biopsy in HNC as well as its future perspectives, and describe both the available evidence for using different liquid biopsy modalities and the upcoming advances in the field [9–14].

2. Current Evidence
2.1. Circulating Tumor Cells (CTC)
2.1.1. CTC in Squamous Cell Carcinoma of the Head and Neck

CTC investigations in patients with HNC have used different methods from one another within small-sample sized studies. Besides, many of them have been limited to CTC counting to use them as a prognostic tool, with only a few studies having sought their molecular characterization.

Most studies have used detection methods based on immunoaffinity with positive or negative enrichment depending on each case. However, a growing number of studies have also been reported with CTC detection methods based on size or other physicochemical characteristics [15–45].

Hristozova et al. [26], using flow cytometry, detected the presence of CTCs in 18/42 (43%) patients with unresectable LA SCCHN, finding that the detection of CTCs correlated with the presence of locoregional lymph node metastases. Gröbe et al. [34], using the CellSearch system, detected CTCs in 12.5% of 110 patients with oral cavity cancer who had undergone R0 surgery, finding that the presence of CTCs was associated with a shorter PFS, a higher T and the presence of locoregional metastases. Tinhofer et al. [27], using a method based on the detection of CTCs through the amplification of tumor antigens, detected CTCs prior to the start of adjuvant treatment in 29% of 144 patients with resected LA SCCHN. The presence of CTCs was higher in tonsil or tongue base tumors, and although there was a non-significant trend towards better disease-free survival (DFS) in oropharyngeal carcinomas, there was a significant association between non-oropharyngeal carcinomas and worse DFS and OS. Finally, Grisanti et al. [28], using the CellSearch™ system, identified CTCs in 26% of 53 patients with R/M SCCHN, finding that the cut-off point of ≥ 1 CTCs was associated with a worse PFS and OS, achieving a significantly higher disease control rate in patients without CTCs than with CTCs (45% vs. 8%, p = 0.03). In the prospective CIRCUTEC study, 65 patients with R/M SCCHN were included, with blood samples being taken at baseline and on the 7th and 21st days after starting the first CT cycle with the EXTREME regimen (cisplatin-5FU-cetuximab three weeks). Three CTC detection systems were used: CellSearch, EPISPOT, and flow cytometry (FCM). EPISPOT, CellSearch and FCM detected CTC in 69%, 21%, and 11% of the cases, respectively. PFS was significantly shorter in patients with stable or increasing CTC numbers between baseline and 7-day determination with EPISPOT (3.9 vs. 6.2 m; 95% CI 5–6.9, p = 0.0103), and the same occurred in patients with CTC ≥ 1 with EPISPOT or with CellSearch (p = 0.03), with EPISPOT or FCM (p = 0.048), and CellSearch or FCM on day 7 (p = 0.0005). This study therefore suggests that CTCs can be used to monitor early response to chemotherapy in R/M SCCHN [31].
In a prospective cohort of 113 patients with LA SCCHN, the CellSearch system was used to determine CTCs at baseline, after 2 cycles of induction CT and at the end of treatment with chemoradiotherapy (CRT). PD-L1 overexpression in CTCs was detected in 25.5%, 23.5% and 22.2% of the patients at each of the moments described, respectively. Patients with PD-L1 overexpression CTCs at the end of CRT had worse PFS ($p < 0.001$) and OS ($p < 0.001$), and it was an independent prognostic factor in multivariate analysis. The authors proposed that PDL1 + CTCs could be used to identify a population of patients that could benefit from adjuvant treatment with checkpoint inhibitors directed against the PD1-PDL1 axis [35]. Along the same lines, Chikamatsu et al. [32] isolated CTCs from 30 patients with R/M SCCHN by negative depletion of leukocyte cells using magnetic particles and the identification of CTCs by mRNA expression of various epithelial markers (CK19, EpCAM, EGFR and c-MET). In addition, the expression in CTCs of PD-L1, PD-L2 and CD47 was analyzed. Positive CTCs were detected for the expression of at least one epithelial marker in 24 cases (80%), and of these, the expression of CD47, PD-L1 and PD-L2 occurred in 79.2%, 83.3% and 70.8%, respectively. There was no correlation between the expression of PD-L1 in the tumor and in the CTCs. In a patient with metastatic squamous cell carcinoma of the larynx, Kulasinghe et al. [33] demonstrated the detection of CTC “clusters” using a spiral microfluidic system, which after staining with specific antibodies were shown to express PD-L1.

This same group compared the CellSearch system with two other non-immunoaffinity-based detection systems, the ScreenCell system and the RosetteSep in patients with SCCHN. CTC were detected in 18.6% (8/43), 46.4% (13/28) and 64% (16/25) of the cases, respectively, and they demonstrated that the two non-IA-based methods detected CTC in a greater number than CellSearch [36].

Morgan et al. [43] used a novel spectroscopy technology which has been used in the development of space research and is and known as Surface-Enhanced Raman Scattering (SERS) nanotechnology. EGF (epidermal growth factor) was used as the capture antigen. First, low-density CTCs and leukocytes were isolated by density gradient centrifugation and were then incubated with SERS-EGF nanoparticles, before being illuminated with a 785 nm laser. The number of CTCs could be determined as a function of the SERS signal intensity. With this system, the number of CTCs detected in 82 patients with LA SCCHN was considerably higher than with other technologies, establishing 675 CTCs as the best cut-off point to predict distant metastasis-free survival (DMFS) at 1, 2 and 5 years.

In a meta-analysis published in 2017, which included a total of 17 studies with CTCs in patients with SCCHN, it was concluded that the presence of CTCs is significantly statistically associated with poor OS (HR 2.8, 95% CI 1.34–5.86), SLE (HR 3.86, 95% CI 2.03–7.36) and PFS (HR 3.31, 95% CI 1.71–6.42). In addition, patients with CTCs tend to relapse more, and present locoregional lymph node metastases and a more advanced T category [44].

The PREDICT-HN (NCT03491176) is an ongoing study in patients with SCCHN who receive treatment with CRT with radical intention and who undergo weekly MRI imaging studies and weekly blood draws for the study of CTCs by the CellSearch and ctDNA system. The main objective is to evaluate the value of tumor response kinetics and CTCs to predict response to treatment [45]. Table 1 summarizes the most relevant studies with CTC in patients with SCCHN.

### 2.1.2. CTC in EBV+ Nasopharyngeal Cancer

Some groups have studied the role of CTCs in patients with NPC [46–56]. Table 2 summarizes the most relevant studies with CTC in patients with NPC.
| Author          | CTC Detection Technology                  | N     | Stage       | N (Samples) | Detection Rate | Prognostic Value |
|-----------------|-------------------------------------------|-------|-------------|-------------|----------------|------------------|
| Wirthshafter (2002) [16] | CellSearch                               | 18    | I-IV        | 18          | 44% (0–3 CTC)  | -                |
| Partridge (2003) [17]     | IA with negative enrichment              | 36    | I-IV        | 36          | 50% (0–5 CTC)  | Worse DFS        |
| Guney (2007) [18]         | CellSearch                               | 21    | LA          | 21          | 33%            | -                |
| Winter (2009) [19]        | ISET (size)                               | 16    | LA          | 32 (pre- and post-SX) | 63% | -                |
| Jatana (2010) [20]        | IA with negative enrichment              | 48    | I-IV        | 61          | 71%            | Worse DFS        |
| Buglione (2012) [21]      | CellSearch                               | 73    | I-IV        | 41 (pre-y post-TX) | 15% (0–43 CTC) | Decrease in CTC → better response |
| Nichols (2012) [22]       | CellSearch                               | 15    | LA          | 15          | 40%            | Worse OS in CTC+ |
| Bozec (2013) [23]         | CellSearch                               | 49 (LA) | LA         | 49 LA SCCHN | 10 (HC) | 16% in SCCHN 0% in N(-) 23% in N1-2c |
| He (2013) [24]            | CellSearch                               | 9     | III-I       | 9           | 33% (0–1 CTC)  | -                |
| Hsieh (2015) [25]         | IA with negative enrichment              | 53    | LA or R/M   | 53          | 19%            | -                |
| Hristozova (2011) [26]    | Fluid Cytometry                          | 42    | Unresectable LA | 42          | 43%            | Association of CTC+ with N+ |
| Große (2013) [34]         | CellSearch                               | 110   | Resected (R0) | 42          | 110            | -                |
| Tinhofer (2014) [27]      | Immunoaffinity through tumor-antigen amplification | 144   | Resected    | 144         | 29%            | Association of CTC+ with <DFS and OS |
| Grisanti (2014) [28]      | CellSearch                               | 53    | R/M         | 53          | 26%            | Association of CTC+ with <DFS and OS |
| Inhesten (2015) [29]      | Fluid Cytometry                          | 40    | II-IV       | 120 (before, during and after TX) | 97% (80% at baseline) | Association of CTC+ with <DFS and OS |
| Dyavanagoudar (2008) [37] | Detection of CK19 with RT-PCR            | 25    | LA OSCC     | 25          | 16%            | -                |
| Kusukawa (2000) [30]      | Detection of CK19 with RT-PCR            | 20    | LA OSCC     | 20          | 10%            | Association of stability/increase of CTC with EPISPOT or CTC+ with CellSearch with <SLP Assocatoin of PDL1+ CTC post-CRT with <PFS and OS |
| Garrel (2019) [31]        | CellSearch, EPISPOT, Fluid Cytometry     | 65    | R/M         | Baseline, d7 and d21 after first cycle of EXTREME | EPISPOT: 69%, CellSearch: 21% CMP: 11% | - |
| Strati (2017) [35]        | CellSearch                               | 113   | LA          | Baseline, after iT and after CRT | PDL1 overexpression in CTC in 25.5% (baseline), 23.5% (after iT) and 22.2% (after CRT) | - |
| Chikamatsu (2019) [32]    | IA with negative enrichment and mRNA expression of epithelial markers (CK19, EpCAM, EGFR, c-MET) | 30    | R/M         | 30          | CTC with epithelial marker expression ≥ 1.80% | - |
| Kulasinghe (2017) [33]    | Spiral microfluidic system               | 1     | R/M         | 1           | Detection of CTC “clusters” with PDL1 expression | - |
| Author                  | CTC Detection Technology                        | N     | Stage | N (Samples) | Detection Rate                                                                 | Prognostic Value                                                                 |
|------------------------|-------------------------------------------------|-------|-------|-------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Kulasinghe (2016) [36] | IA vs. NIA: CellSearch (IA) vs. ScreenCell (NIA) vs. RosetteSep (NIA) | 43    | R/M   | 43          | CellSearch: 18.6% (8/43) ScreenCell: 46.4% (13/28) RosetteSep 64% (16/25)       | Detection of CTC with epithelial phenotype (E-CTC) in 75% and CTC with mesenchymal phenotype (M-CTC) in 90% Association of M-CTC with higher odds of distant metastases and shorter PFS |
| Liao (2019) [38]      | IA with negative enrichment                    | 20    | LA or R/M | 20          | Detection of CTC with epithelial phenotype (E-CTC) in 75% and CTC with mesenchymal phenotype (M-CTC) in 90% Association of CTC with N+, PR vs. CR and <DFS cDFS and OS and a higher CTC count Association of CTC reduction with longer PFS and OS Association of higher number of CTC with more advanced stage |
| Zheng (2019) [39]     | CytoSorter (IA + microfluidic system)           | 20 (LA) 6 (LTB) 12 (HC) | LA     | Pre- and post-iCT | HC: 0% CTC+ SCCHN: 77% CTC+ Association of CTC with N+, PR vs. CR and <DFS cDFS and OS and a higher CTC count Association of CTC reduction with longer PFS and OS Association of higher number of CTC with more advanced stage |
| Chang (2019) [40]     | IA with negative enrichment                    | 34    | R/M   | 34          | -                                                                                | Association of higher number of CTC with more advanced stage                     |
| Wang (2019) [41]      | IA with negative enrichment                    | 53    | LA    | Before and during CRT | -                                                                                | Association of higher number of CTC with more advanced stage                     |
| Kawada (2017) [42]    | CellSieve (Low pressure microfiltration system) | 32    | R/M   | 32          | 90.6%                                                                            | Association of higher number of CTC with more advanced stage                     |
| Onidani (2019) [15]   | LFIMA (microfluidic and inertial detection system) vs. ctDNA | 9     | R/M   | 9           | Mutations in CTC in 4/9 pts and in ctDNA in 1/9 pts Association of higher number of CTC with DMFS Association of higher number of CTC with <PFS and <OS |
| Morgan (2019) [43]    | SERS (RAMAN-type spectroscopy)                 | 82    | LA    | 82          | -                                                                                | Association of higher number of CTC with more advanced stage                     |
| Sun (2017) [44]       | Meta-analysis of 17 CTC studies in SCCHN       | -     | -     | -           | -                                                                                | Association of higher number of CTC with more advanced stage                     |

SCCHN: head and neck squamous cell carcinoma, CR: complete response,CK19: cytokeratin 19, CO: oral cavity, CS: healthy controls, CTC: circulating tumor cells, ctDNA: circulating tumor DNA, DMFS: distant metastasis-free survival, DFS: disease-free survival, FCM: flow cytometry, HPV: human papillomavirus, IA: immunoaffinity detection method, ISET: isolation by size of epithelial tumor cells, LA: locally advanced disease, n: number, N: locoregional cervical nodes, NIA: non-immunoaffinity detection method, OS: overall survival, OSCC: oral squamous cell carcinoma, PFS: progression-free survival, PR: partial response, R0: complete tumor resection with free margins, R/M: recurrent and/or metastatic phase, RT-PCR: real-time PCR, SX: surgery, TBI: time to control of disease, TX: therapy.
Table 2. Most relevant studies with CTC in patients with EBV+ NPC.

| Author       | CTC Detection Technology | \( N \) (Patients) | Stage | N (Samples) | Detection Rate | Prognostic Value                                                                 |
|--------------|--------------------------|---------------------|-------|-------------|----------------|----------------------------------------------------------------------------------|
| Zhang (2018) [46] | SE-iFISH                | 50                  | II-IV | Pre- and post-CT | 92%            | Higher CTC count with a higher TNM/AJCC stage                                   |
| Zhang (2018) [46] | ISET                 | 33                  | I-IV  | 33          | 66.7%          | CTC count correlated with tumor response                                         |
| Si (2016) [47]     | CanPatrol™ (size detection method) EBV-ctDNA detected with RT-PCR | 81                  | I-IV  | 81          | 66.4% COX2+ CTC at baseline           | Good correlation of CTC count with EBV ctDNA                                    |
| You (2019) [48]   | CTC vs. EBV-ctDNA       | 148 R/M             | 122 LA | Pre- and post-CT | 96.6%          | Higher CTC count correlated with N+ and M1 disease                               |
| Li (2018) [49]     | CTC and COX-2 expression in CTC | 131                | LA     | Pre- and post-CRT | 66.1% COX2+ CTC post-CRT | CTC and EBV-ctDNA have prognostic value that increases when combined between them and with tumor imaging tests |
| Vo (2016) [50]     | CTC (Microsieve) vs. EBV-ctDNA (qPCR and dPCR) | 46                  | LA     | Pre- and post-CRT | 66%             | < COX-2 expression post-CRT                                                    |
| He (2017) [51]     | ISET + IHC CK5/CK6/P36 ISET + I SH EBERs mRNA-hTERT in plasma and in CTC | 33                  | LA     | Baseline     | 66.7%           | Correlation of CTC count and titles of EBV VCA-IgA and EBV-ctDNA                |
| Fu (2017) [52]     | mRNA-hTERT in plasma and in CTC | 33 NPC              | 24 HC  | Pre- and post-CRT | -               | Association of mRNA-hTERT in plasma and CTC with clinical stage and response to therapy |
| Wen (2019) [55]    | CanPatrol technology    | 60 NPC              | 18 HC  | LA           | CTC+: 86.7% CTCmesenq+: 50%          | Reduction of CTC count with ICT                                                   |
| Sun (2019) [54]    | CellSpotter Analyzer vs. EBV-ctDNA (RT-qPCR) | M0: 114, M1: 136    | Baseline | Median number of CTC: | M0: 9.3 M1: 12.5 | Worse prognosis with CTCmesenq+                                                  |
| Ou (2019) [53]     | CellSearch             | 370                 | III-IV | Baseline    | CTC+: 77/288 (27%) M1: 16/81 (20%) | CTC and LMP1, BART and EBER1 levels higher in M1 vs. M0                         |
| Xie (2019) [56]    | CanPatrolTM + HIS (COX-2) vs. EBV-ctDNA (RT-qPCR) | 50 II-IV, 50 NPC     | II-IV  | 10 HC        | CTC+: 96% M-CTC+: 76%             | Worse prognosis with higher CTC count                                             |

AJCC: American Joint Committee on Cancer, CK: cytokeratin, CRT: chemoradiotherapy, CT: chemotherapy, Cr: chromosome, ctDNA: circulating tumor DNA, dPCR: digital PCR, mRNA: messenger RNA, CTC: circulating tumor cells, EBERs: EBER1 and EBER2 are EBV noncoding RNAs, EBV: Epstein-Barr virus, HC: healthy controls, iCT: induction chemotherapy, IHC: immunohistochemistry, ISET: Isolation by Size of Tumor Cells, I/L/LA: localized/locally advanced phase, MMP-9: matrix metalloproteinase 9, M0: non-metastatic stage, M1: metastatic stage, Mesenq: mesenchymal, NPC: nasopharyngeal cancer, n: number, N: locoregional cervical nodes, qPCR: quantitative PCR, RT-PCR: real-time PCR, SE-iFISH: subtraction enrichment and immunostaining-fluorescence in situ hybridization, TNM: tumor, node, metastasis classification.
Zhang et al. [46], in a cohort of 50 patients with NPC stage II-IV, used the SE-iFISH technology for CTC detection before and after chemotherapy; they achieved a 92% CTC detection rate and demonstrated a higher CTC detection in more advanced stages. A decreased CTC count and chromosome 8 aneuploidy in CTC after chemotherapy was associated with tumor response. The same group used ISET technology in 33 stage II-IV NPC patients to demonstrate a 66.7% detection rate with a good correlation with EBV viral load [46]. Si et al. [47], using the CanPatrol™ system in 81 stage I-IV NPC patients, demonstrated a detection rate of 96.6% and a higher CTC count in N+ and M1 disease.

You et al. [48] compared CTC versus EBV-ctDNA pre- and post-chemotherapy in 148 R/M NPC patients and in 122 LA NPC patients, demonstrating that the prognostic role of both CTC and ctDNA improved when both are used together and when combined with imaging tests.

Li et al. [49] studied CTC detection and COX-2 expression in CTC in 131 LA NPC patients, demonstrating a 66.4% and 46.1% COX2+ CTC detection rate before and after chemoradiotherapy, respectively. In the latter case, this was associated with a worse prognosis.

Ou et al. [53], using the CellSearch system in 370 patients with stage III-IV NPC, detected CTC in 27% and 20% of non-metastatic and metastatic patients, respectively, demonstrating a worse prognosis with a higher CTC count. Combining CTC and EBV-ctDNA improved their prognostic value.

Sun et al. [54] compared EBV-ctDNA versus CTC detection with the CellSpoter Analyzer technology in 144 non-metastatic and 136 metastatic NPC patients, and found CTCs in 9.3% and 12.5%, respectively.

Several other smaller studies have also demonstrated the prognostic value of CTC in patients with NPC, and these are summarized in Table 2 [46–56].

2.1.3. CTC in Salivary Gland Cancer

Very few studies have been published regarding the use of liquid biopsy in salivary gland cancers, despite their frequently redundant hotspot alterations in certain entities such as salivary duct carcinoma (SDC), mucoepidermoid carcinoma (MEC), NTRK-driven mammary analog secretory carcinoma (MASC) or adenoid cystic carcinoma (ACC). Common alterations include HER2 amplification, androgen receptor (AR) overexpression, MYC amplification, PIK3CA mutations and NTRK fusions [57–60]. In a pilot study of 8 patients with ACC using a spiral microfluidics-based isolation method and detection by immunofluorescence, 3 patients (32.5%) were positive for CTC, each patient with either metastatic or recurrent disease [61]. In a case reported by Cappelletti et al. [62], in a patient with metastatic SDC, using the CTC-enrichment method Parsortix followed by single-cell identification and recovery with the DEPArray technology demonstrated the AR splicing variant 7 (Arv7) in CTC and predicted the development of resistance to abiraterone therapy 6 months in advance.

2.1.4. CTC in Paranasal Sinus Cancer

Paranasal sinus cancers encompass a diverse family of entities ranging from squamous cell carcinomas to adenocarcinomas and undifferentiated carcinomas. Among adenocarcinomas, two main reference types exist: intestinal-type adenocarcinomas (ITAC) and non-ITAC type. Unfortunately, there is a major lack of evidence on the use of liquid biopsy in paranasal sinus cancer. Our group published the case of a patient with recurrent ITAC in whom liquid biopsy using the IsoFlux EPCAM-based microfluidic technology detected 26 CTC. Genomic analysis of CTC DNA using castPCR revealed a mutation in KRAS G12A that was retrospectively also detected in the primary tumor using BEAMing technology [63].
2.2. Circulating Tumor DNA (ctDNA)

2.2.1. ctDNA in HPV-Unrelated Squamous Cell Carcinoma of the Head and Neck

Lin et al. [64] studied ctDNA in 121 patients with oral cavity SCCHN and in 50 healthy controls. The level of ctDNA was significantly higher in patients with SCCHN than in controls, and it was correlated with tumor size, the presence of regional lymph node metastases and with more advanced stages. Perdomo et al. [65] studied mutations in ctDNA in 5 genes (TP53, NOTCH1, CDKN2A, CASP8, PTEN) using NGS, in 36 patients with stages II-IV SCCHN. In 42% of the cases, mutations were detected in the ctDNA, and 67% of these corresponded to stage I and II patients. A total of 18 matching mutations were detected between plasma and the primary tumor. In another series of 37 patients with stages III and IV SCCHN, complete TP53 sequencing was performed in tumor, plasma and oral rinse samples, as well as in 49 healthy controls. Thirty-six TP53 mutations were detected in tumor, three in plasma, and twenty-six in oral rinses. In 67%, at least 1 mutation in TP53 was detected in the tumor. The concordance of TP53 mutations between tumor, mouthwashes (11%), and plasma (2.7%) was low. The proportion of positive cases in oral rinses was higher in tumors of the oral cavity or oropharynx than in tumors of the larynx. Fostira et al. [66], using the SafeSEQ NGS technology, found a 51% and 93% tumor-plasma concordance in LA and R/M SCCHN, respectively.

Another very promising application of ctDNA is the detection of minimal residual disease (MRD) after curative-intent therapy. Flach et al. [67], in a single-center prospective cohort study used whole exome sequencing of FFPE samples and compared the mutational profile with that acquired from pre- and post-surgical plasma samples analyzed through deep sequencing using RaDaR TM technology. Among 17 patients, ctDNA was detected in all of the patients prior to surgery, and only in very low levels in post-surgery samples. In all cases with clinical relapse, ctDNA was positive between 108 and 253 days post-surgery. This approach demonstrates the feasibility of using personalized ctDNA assays for disease detection prior to clinical relapse.

Blood tumor mutational burden (bTMB) was analyzed in patients from the EAGLE trial, a second-line trial comparing standard chemotherapy versus the anti-PDL1 durvalumab alone or versus durvalumab plus the anti-CTLA4 tremelimumab. Although the study did not achieve its primary endpoint for a longer OS for any of the immunotherapy arms, in a subgroup analysis, a high-bTMB (≥16 muts/mb) predicted a better OS and PFS for patients treated with immunotherapy compared to chemotherapy. In addition, ctDNA positive for mutations in KMT2D or ATM predicted a better OS for patients treated with durvalumab plus tremelimumab versus standard chemotherapy [68].

Porter et al. [69], in a study of 60 patients with head and neck cancer using the Guardant360 NGS technology, detected ctDNA alterations in plasma in 66% of patients with SCCHN and in 50% of patients with salivary gland cancer (SGC). TP53 (68%), PIK3CA (34%), NOTCH1 (20%) and ARID1A (15%) were the most common alterations detected in ctDNA. These results were conondant with tumor NGS, although 73% had blood alterations not identified in tissue. Wilson et al. [70], in a study of 75 patients with R/M SCCHN, actionable ctDNA alterations were detected in 65% of the patients, although tumor-ctDNA concordance was only 13%. ctDNA detection was associated with the extent of disease and with a worse OS.

These and other smaller studies are summarized in Table 3 [64–72].
Table 3. Most relevant studies with ctDNA in patients with HPV-unrelated SCCHN.

| Author            | N                  | Setting          | Technology                          | Detection Rate | Specificity | Conclusions                                                                 |
|-------------------|--------------------|------------------|-------------------------------------|----------------|-------------|-----------------------------------------------------------------------------|
| Lin (2018) [64]   | 121 SCCHN (OC) 50 | LA               | dPCR                                | -              | -           | ctDNA level higher in SCCHN vs. HC                                          |
|                   | SCCHN              |                  |                                     |                |             | Higher levels of ctDNA with higher T stage and N stage                     |
|                   |                    |                  |                                     |                |             | Reduction in levels of ctDNA in 75% of cases after surgical resection       |
| Van Ginkel (2017) [71] | 6 SCCHN         | LA               | dPCR                                | 100%           | 100%        | Detection of mutations in 100% of cases                                     |
| Egyud (2019) [72] | 8 SCCHN            | LA               | NGS                                 | 6/8            |             | Relapse in 4/8 pts, in 2 of them ctDNA detectable before relapse            |
| Perdomo (2017) [65] | 36 SCCHN plasma | LA               | NGS (TP53, NOTCH1, CDKN2A, CASP8, PTEN) | 18/36 (67%) (stages I and II) | -           | Detection of 18 concordant mutations between primary tumor and plasma      |
| Perdomo (2017) [65] | 37 SCCHN plasma & oral rinses | LA | NGS (TP53) | 3/37 | - | Low concordance of TP53 mutations between the primary tumor, plasma, and oral washings |
| Fostira (2019) [66] | 54 LA SCCHN 15 R/M SCCHN | LA and R/M | SAFESEQ (TP53, CDKN2A, PIK3CA, HRAS) | LA: 51% | R/M: 93% | - High tumor-plasma concordance, especially in R/M |
|                   |                    |                  |                                     |                |             | - Detection of emerging mutations during treatment or after PD            |
| Flach (2022) [67] | 17 LA SCCHN       | LA               | FFPE: WES Plasma: NGS deep sequencing (RaDaR™) | Pre-SX: 100% | Post-SX: | High tumor-plasma concordance in pre-SX samples |
|                   |                    |                  |                                     |                |             | All relapses showed post-SX ctDNA positive samples                        |
| Li (2020) [68]    | 247 R/M SCCHN (2nd line) | R/M | NGS Pre-TX | - | - | High bTMB (≥16 muts/Mb) predicted a longer OS and PFS with IO. |
| Porter (2020) [69] | 60 R/M HNC 21 OPC, 8 SGC, 4 Thyroid cancer | R/M | NGS (Guardant360) | SCCHN: 66% SGC: 50% Thyroid: 100% | - | Most common mutations in plasma: TP53 (68%), PIK3CA (34%), NOTCH1 (20%), ARID1A (15%). These results were concordant with tumor NGS, although 73% had blood alterations not identified in tissue. |
|                   |                    |                  |                                     |                |             | Alterations found in ctDNA allowed to inform management decisions.       |
| Wilson (2021) [70] | 75 R/M SCCHN      | R/M               | NGS                                 | 65% with actionable ctDNA alterations | - | Concordance among altered genes between tumor and ctDNA was 13% |

CT: chemotherapy, ctDNA: circulating tumor DNA, dPCR: digital PCR, FFPE: formalin-fixed parafin-embedded, IO: immunotherapy, LA: locally advanced disease, HC: healthy controls, N: number, NGS: next generation sequencing, OC: oral cavity, OS: overall survival, PD: progression, PFS: progression-free survival, R/M: recurrent and/or metastatic phase, SCCHN: squamous cell carcinoma of the head and neck, SX: surgery, WES: whole exome sequencing.
In a systematic review of 16 studies from 4 different countries including 1156 patients and 601 controls, ctDNA methylation was significantly increased in patients with SCCHN. The most frequently studied gene mutations were those in TP53 and the most frequently studied gene methylations were those in CDKN2A, DAPK1, RASSF1 and P15 [73]. Similarly, in another systematic review of 10 studies including 390 samples from patients with SCCHN and 79 control samples, the most studied mutation was TP53 [74].

2.2.2. ctDNA in HPV-Related Squamous Cell Carcinoma of the Head and Neck

There are several recent studies that demonstrate the usefulness of the ctDNA study of specific sequences of the human papillomavirus (HPV). Most studies have been carried out in non-advanced disease, either localized or locally advanced, with detection rates of ctDNA in plasma ranging from 65% to 96%, and also with high specificities, generally between 89% and 100% [6,75–80].

Damerla et al. [75], using digital PCR in 97 patients with HPV(+) localized OPC, detected HPV-ctDNA in plasma in 95.6%. Chera et al. [76], using digital PCR in 103 patients with HPV(+) localized OPC, detected HPV-ctDNA in plasma in 89% of the patients. Interestingly, 35% of the patients with <95% clearance of HPV-ctDNA after chemoradiotherapy relapsed, while there were no relapses in those with ≥95% clearance.

Cao et al. [78], in 40 patients with HPV-related localized OPC, demonstrated a 65% plasma ctDNA detection rate. Plasma HPV-ctDNA progressively increased in the 3 patients with metastatic relapse. Dahlstrom et al. [77], in 262 HPV-related localized OPC, showed a 60.5% HPV-ctDNA detection rate and 67% specificity using RT-PCR in plasma. Baseline HPV-ctDNA was associated with the nodal and global stage. Ahn et al. [79] demonstrated in 52 patients with HPV(+) OPC that the detection of HPV-ctDNA in plasma or in saliva after concomitant CRT was associated with a high positive predictive value; those ctDNA-HPV+ patients in plasma or saliva had a relapse-free survival (RFS) and overall survival (OS) significantly shorter than the ctDNA-negative patients in saliva and plasma.

Mazurek et al. [80], in a large cohort of HPV-related OPC, demonstrated a 14% HPV-ctDNA detection rate using RT-PCR in plasma, with a higher plasma ctDNA quantity in HPV-related compared to HPV-unrelated cancers.

In another study conducted in 21 patients with HPV(+) and HPV(−) SCCHN, HPV DNA sequences and frequent somatic mutations in head and neck cancer were studied in plasma and saliva [6]. The saliva study detected alterations in ctDNA in 100% of oral cavity cancers (OCC), 47% of OPC, 70% of laryngeal cancers and 67% of hypopharyngeal cancers, while the plasma detection rates at these subsites were 80%, 91%, 86% and 100%, respectively. However, when plasma and saliva detection were combined, the identification of molecular alterations in ctDNA occurred in 100% of OCC, 91% of oropharyngeal cancers and in 100% of cancers of the larynx and hypopharynx.

Siravegna et al. [81], in a prospective observational study of 70 patients with SCCHN and 70 healthy controls, analyzed plasma ctDNA with ddPCR for HPV genotypes 16, 18, 33, 35 and 45. Sensitivity and specificity of ctDNA was 98.4% and 98.6%, respectively, demonstrating a higher diagnostic accuracy than the standard physical examination and imaging. Costs of HPV ctDNA detection were 36–38% less and ctDNA diagnosis occurred a median of 26 days earlier than with the standard clinical workup. The same group, in another study of 33 patients with resected HPV+ OPC, found that in patients without pathologic risk factors for recurrence, HPV-ctDNA rapidly decreased at postoperative day (POD) 1. However, in patients with risk factors for macroscopic disease, HPV-ctDNA was overtly elevated at POD 1. This elevation was maintained until the start of adjuvant treatment. Higher levels of HPV-ctDNA were detected with extranodal extension > 1 mm and with an increasing number of affected lymph nodes. Therefore, HPV-ctDNA could aid in individualizing adjuvant treatment after surgery in HPV+ OPC [82].

Akashi et al. [83], in a study of 25 patients with HPV+ OPC, detected HPV-specific ctDNA in plasma in 14 (56%) patients at baseline, and found that this was negative after therapy in all patients. In 2 patients, HPV-specific ctDNA was detected at the time of recurrence (Table 4).
| Author (Year) | N | Setting | Technology | Detection Rate | Specificity | Conclusions |
|---------------|---|---------|------------|----------------|-------------|-------------|
| Damerla (2019) [75] | 97 HPV(+) SCCHN (OPC) 6 HPV(−) SCCHN 20 HC | Localized | dPCR | 95.6% | 100% | ctDNA detected HPV16 and 33 with same accuracy that in tissue HPV-ctDNA also detectable in small tumors None of the pts with ≥ 95% of ctDNA clearance relapsed 35% of the pts with <95% of ctDNA clearance relapsed HPV-ctDNA should be explored as a marker for deintesification strategies in HPV(+) disease Higher HPV-ctDNA levels in OPC vs. other locations |
| Chera (2019) [76] | 103 SCCHN | Localized | dPCR | 89% | 97% | Higher HPV-ctDNA levels with higher stages Similar ctDNA levels in HPV(+) and HPV(−) SCCHN |
| Mazurek (2016) [80] | 200 SCCHN (HPV(+) and HPV(−)) | Localized | RT-PCR (TERT amplification and HPV16/HPV18) | 14% HPV+ in plasma | - | Higher HPV-ctDNA levels in OPC vs. other locations |
| Wang (2015) [6] | 93 SCCHN | Localized | RT-PCR (E6 y E7), PCR multiplex (E6 y E7) HPV(−): NGS (TP53,PIK3CA, CDKN2A,FBXW7, HRAS, NRAS) | L/ LA: 10/10 (100%) R/M: 37/39 (95%) Saliva: OC (100%), Other (47–70%) Plasma: OC (80%), Other (86%) | - | High detection rate in plasma and saliva ctDNA detection in 3/3 cases that relapsed and in 0/5 cases that did not relapse |
| Dahlstrom (2015) [77] | 262 SCCHN | Localized (I-IV) | RT-PCR | 60.5% | 67% | Baseline HPV-ctDNA associated with global and N stage Baseline HPV-ctDNA was not a predictor of relapse |
| Cao (2012) [78] | 40 HPV(+) 24 HPV(−) 10 HC | Localized | RT-PCR | 65% | - | HPV-ctDNA negativization after RT in 14 pts Increase in HPV-ctDNA in 3 pts with metastatic relapse |
| Ahn (2014) [79] | 93 plasma and saliva pre- and post-TX (81 HPV(+) y 12 HPV(−)) | Localized | RT-PCR | 67% | 89% | OS shorter in pts with detectable HPV-ctDNA post-TX in plasma or saliva |
| Siravegna (2021) [81] | 61 HPV(+) SCCHN 45 HPV(−) SCCHN 25 HC | LA newly diagnosed SCCHN | ddPCR (HPV 16,18,33,35, 45) | 98.4% | 98.6% | Very high detection rates, with lower cost and earlier diagnosis compared to standard clinical workup |
| O’Boyle (2022) [82] | 33 | L/ LA treated with surgery | ddPCR (HPV 16, 18, 33, 35, 45) | - | - | ctDNAHPV levels on POD 1 were associated with residual disease 56% detection rate at baseline. 0% detection rate after treatment. In 2 relapsing patients, HPV-specific ctDNA was positive. |
| Akashi (2022) [83] | 25 HPV(+) | L/ LA newly diagnosed SCCHN | dPCR (E6 & E7 regions of HPV DNA) | 56% | - | |

ctDNA: circulating tumor DNA, dPCR: digital PCR, ddPCR: droplet digital PCR, HC: healthy controls, HPV: human papillomavirus, L: localized disease, LA: locally advanced, OC: oral cavity, OPC: oropharyngeal cancer, POD: postoperative day, RT: radiotherapy, RT-PCR: real-time PCR, SCCHN: head and neck squamous cell carcinoma, TX: therapy.
2.2.3. ctDNA in EBV+ Nasopharyngeal Cancer (NPC)

The study of ctDNA in the plasma of EBV+ NPC began nearly 2 decades ago. There are, therefore, several authors who have demonstrated the feasibility of studying the ctDNA of specific regions of EBV in the plasma of patients with NPC, both as a prognostic tool that allows refining the TNM staging system and as a variable that allows the identification of patients who would benefit from more intensive treatment with induction chemotherapy followed by radical CRT instead of radical CRT alone [84–91]. It has even been used in the screening of NPC in an endemic population. In a study carried out in Hong-Kong, 20,349 people were screened, 309 of whom were identified as having detectable levels of EBV-ctDNA in their plasma, initiating a screening program directed by nasofibroscopy and magnetic resonance imaging (MRI) which allowed 34 patients to be diagnosed with NPC, most of whom were in the early stages (stages I and II AJCC 7th Ed.). The survival rate achieved compared favorably with the overall number of patients diagnosed in 2013 in the same area, the majority in stages III and IV (AJCC 7th Ed) [92].

Our group recently published the evolution of two cases of patients with metastatic NPC who, after progressing to treatment with immunotherapy with the anti-PD1 agent nivolumab, were rescued with platinum-gemcitabine, and where the dynamics of EBV-ctDNA in plasma correlated with the radiological response, demonstrating its role for the monitoring of advanced disease [93]. See Table 5.

2.3. Extracellular Vesicles

Extracellular vesicles (EVs) are non-nucleated lipid-layered round elements liberated from normal cells but also from tumor cells; they are implicated in multiple biological processes and harbor a rich protein and genetic (RNA and DNA) cargo, constituting an important reservoir of molecular information from their parental cells [9,10]. Tumor exosomes (TEX) may be isolated through different methods such as their biophysical properties or immune affinity, although ultracentrifugation is one the most used methods [4]. TEX are 30–150 nm EVs that are produced in high quantities by several solid tumors and are enriched in the plasma of patients with cancer, including SCCHN. Among their different roles, they perform major immunosuppressive functions in the tumor microenvironment [9]. Theodoraki et al. [94], in a study of 40 SCCHN patients, found that PD-L1+ TEX correlated with nodal and disease stage, while soluble PD-L1 in plasma did not. CD69 expression levels on T cells were inhibited by incubation with PD-L1high TEX. Interestingly, in-vitro blockade of PD-L1+ TEX signaling to T cells diminished immune suppression. Recently, the same group, in a cohort of 17 SCCHN patients treated with surgery followed by adjuvant (chemo)radiation, demonstrated tumor exosomal protein decrease and tumor-/immune-cell derived exosomes decrease in responders, but an increase at the time of relapse, representing a promising liquid biopsy modality for early detection of relapse [95]. Among 18 SCCHN patients enrolled in a phase I trial and treated with cetuximab, ipilimumab and radiotherapy serial blood samples were collected for TEX and T cell-derived exosomes. While in patients who remained disease free, total exosome protein and TEX levels decreased, in patients with disease relapse, total exosomes protein and TEX levels increased [10]. Among 9 SCCHN treated with photodynamic therapy (PDT) blood samples were collected before and at 3 timepoints after therapy. TEX obtained before and 24 h after PDT were enriched in N-cadherin and TGF-β1 and enhanced tumor proliferation, migration and invasion. On the other hand, TEX from day 7 or from 4–6 weeks after PDT were enriched in E-cadherin and restored epithelial morphology and EpCAM expression in tumor cells, reduced the expression of mesenchymal genes and inhibited proliferation, migration and invasion, suggesting that PDT promotes the transition from a mesenchymal to an epithelial tumor phenotype mediated by TEX [96]. Wang et al. [97] compared the detection of miR-21 and HOTAIR in TEX between 52 patients with laryngeal SCCHN and 49 patients with polyps of the vocal cords. Levels of miR-21 and HOTAIR within TEX were significantly higher in patients with laryngeal carcinoma compared to patients with vocal cord polyps. Likewise, these miR-21 and HOTAIR levels within TEX were higher in laryngeal SCCHN patients with lymph node metastasis compared to those without.
Table 5. Most relevant studies with ctDNA in patients with EBV+ NPC.

| Author          | N       | Setting          | Technology | Detection Rate | Conclusions                                                                 |
|-----------------|---------|------------------|------------|----------------|------------------------------------------------------------------------------|
| Chen (2018)     | 385     | Stage II NPC     | RT-qPCR    | 161/385 (41.8%) | EBV-ctDNA levels and tumor volume allows to classify stage II NPC into favorable and unfavorable prognostic groups |
| Zhang (2015)    | 1467    | Stage I-IVB NPC  | RT-qPCR    | -              | EBV-ctDNA levels complement TNM improving its prognostic value               |
| Guo (2019)      | 1529    | Stage I-IVA NPC  | RT-qPCR    | -              | EBV-ctDNA levels complement TNM improving its prognostic value               |
| Lee (2019)      | 518     | Stage I-IVA NPC  | RT-PCR     | Median baseline EBV-ctDNA: 588 copies/mL | EBV-ctDNA levels complement TNM 8th Ed improving its prognostic value        |
| Liu (2015)      | 185     | Stage III-IVA NPC| RT-qPCR    | Pre-iCT: 89% Post-iCT: 31% | Detectable EBV-ctDNA post-iCT associate with a worse Px                     |
| Xu (2019)       | 2692    | Stage III-IVA NPC| RT-qPCR    | Pre-iCT EBV-ctDNA ≥ 2000 copies/mL: 57.5% | High levels of EBV-ctDNA pre-iCT associate with a worse Px and identify a group that benefits from iCT |
| Huang (2019)    | 278     | Stage III-IV NPC | RT-qPCR    | Pre-iCT median EBV-ctDNA levels: 9035 copies/mL Post-iCT: 23.7% | High levels of EBV-ctDNA post-iCT associate with a worse Px                 |
| Zhang (2018)    | 4482    | Stage III-IVB NPC| RT-qPCR    | Median EBV-ctDNA: 3740 copies/mL | High levels of EBV-ctDNA before iCT identified a poor-prognosis group that benefits from iCT |
| Chan (2017)     | 20,349  | Stage I-IVB NPC  | RT-qPCR    | Screening: 309/309 (5.5%) | Screening in an endemic population allowed to augment the % of detected cases in early stage (I-II) and this associated with a better survival. |
| Cabezas-Camarero (2020) | 2   | Stage IV NPC     | RT-qPCR    | 100%            | Levels of EBV-ctDNA associated with response to CT post-IO                  |

CT: chemotherapy, ctDNA: circulating tumor DNA, EBV: Epstein-Barr virus, HC: healthy controls, iCT: induction chemotherapy, IO: immunotherapy, LA: locally advanced disease, n: number, NGS: next generation sequencing, NPC: nasopharyngeal carcinoma, Px: prognosis, R/M: recurrent and/or metastatic phase, RT-PCR: Real-time PCR, RT-qPCR: Real-time quantitative PCR, TNM: tumor, node, metastasis classification.
2.4. MicroRNAs

MicroRNAs (miRNAs) are single-stranded non-coding RNAs of 18–25 nucleotides in length. They bind to the 3′-UTR of messenger RNAs, thereby regulating gene expression at the post-transcriptional level by means of RNA degradation and/or translational inhibition. They are highly stable at high temperatures or low pH and are involved in multiple biological processes during cancer development such as cell proliferation, invasion and metastasis. Previously, miRNAs have been detected in different body fluids, including serum, plasma and saliva in patients with SCCHN, and might be a promising liquid biopsy modality in this disease [97–99].

Interestingly, in a study evaluating the role of miR-196a and miR-196b in the early detection of oral cancer, levels of these markers were analyzed in 53 healthy controls, 16 patients with pre-malignant lesions and 90 patients with oral cancer. The miR-196a and miR-196b in plasma were significantly upregulated in patients with oral premalignant lesions and in patients with oral cancer compared to healthy controls. The combined determination of miR-196a and miR-196b showed very high sensitivity and specificity for the diagnosis of patients with oral premalignant lesions and oral cancer [100].

In another study, the levels of miR-31 were analyzed in saliva and plasma of 45 patients with oral carcinoma, 10 patients with oral verrucous leukoplakia and 24 healthy controls. Salivary miR-31 was increased in patients with oral carcinoma compared to controls, but there were no differences between patients with oral leukoplakia and healthy controls. In addition, miR-31 levels were higher in saliva than plasma, and salivary miR-31 levels were significantly reduced in patients with oral cancer after tumor resection [101].

Among 41 patients with head and neck cancer, 66% of whom had NPC and locally advanced disease, long non-coding RNAs (lncRNAs) lncRNA-p21, GAS5 and HOTAIR were analyzed in peripheral blood before and 4.5 months after therapy. Pretreatment GAS5 levels were significantly higher in patients with partial response/progressive disease compared with those achieving a complete response [102].

A meta-analysis identified miR-21 and miR-93 as the most upregulated miRNAs in SCCHN, while miR-9, miR-203, miR-375 are commonly downregulated [98].

A recent meta-analysis of 17 studies including 759 subjects demonstrated that miRNAs detected in saliva of patients with SCCHN had a 69.7% sensitivity and a 86.8% specificity, with an area under the curve (AUC) of 0.803 and a high diagnostic odds ratio (DOR) reaching 12.915 [99].

Finally, another meta-analysis of 25 studies including 2562 subjects with oral cavity cancer found a 78% and 82% diagnostic sensitivity and specificity, respectively, for blood and salivary miRNAs, with an AUC of 0.91 and a high DOR of 21.46 [103].

3. Future Perspective

The definitive implementation of liquid biopsy in HNC requires the clinician to have knowledge of two fundamental aspects: the molecular biology of cancer and the biological and biotechnological foundations for the use of each liquid biopsy modality. The future of liquid biopsy will be shaped by the joint use of different modalities and their combination with tumor imaging tests to provide more sensitive and refined information for clinical practice. It is probable that ctDNA will become the ideal tool in the short-midterm for the diagnosis, monitoring and measuring of MRD in viral-associated head and neck cancers such as HPV-related OPC and EBV-positive NPC. However, further prospective specifically designed studies are needed to demonstrate their role in treatment de-escalation strategies [65,67,70,78,94].

CTCs and EVs constitute some of the sources of molecular information with the greatest potential, as they allow multiple biological components to be analyzed, in contrast to ctDNA or miRNAs. However, important advances are still required to simplify their study and reduce costs [5]. The study of specific metabolites and metabolic signatures have shown interesting results in a few studies in patients with cancer, including SCCHN, and may demonstrate a promising role in a near future [4].
The role of saliva as the biological sample to be liquid biopsied is on the rise and will probably show promise as a screening strategy in premalignant or early malignant oral cavity and oropharyngeal cancers [6,104].

Finally, there is a potential role for liquid biopsy, probably in association with telemedicine, in early-disease diagnosis and monitoring, especially in resource-limited settings, since a well-organized and centralized web-like infrastructure for sample collection and rapid ctDNA analysis could serve as an initial screening allowing to tailor further and more complex investigations [105].

Several ongoing observational studies are evaluating the role of liquid biopsy in HNC and are summarized in Table 6.
Table 6. Current ongoing studies evaluating the role of liquid biopsy in head and neck cancer.

| ClinicalTrials.gov (Accessed on 15 May 2022) (Other Study IDs) | Design | N | Sample Type | Primary Endpoint | Secondary Endpoint | Enrollment Status |
|---------------------------------------------------------------|--------|---|-------------|------------------|--------------------|------------------|
| NCT05122507 (KOHACIN study)                                   | Prospective cohort study | 200 | Blood and saliva | Early recurrence detection lead time (time between liquid biopsy-based recurrence detection and clinical recurrence or progression) | RFS, OS | Recruiting |
| NCT03942380                                                   | Prospective cohort study | 500 | Blood (ctDNA, RNA, HPV-ctDNA) | % of HNC (all histologies) detected using liquid biopsy in blood | % of HNC (all histologies) recurrence detected using liquid biopsy in blood | - | Recruiting |
| NCT03702309 (LIBERATE study)                                  | Prospective cohort study | 2500 (Several cancer types including HNC) | Archived tissue and peripheral blood | Collection and annotation of biospecimens at the Princess Margaret Cancer Center | Implement an electronic informed consent process for clinical research and correlative studies questionnaire at the Princess Margaret Cancer Center | Active, not recruiting |
| NCT04606940 (IO-KIN)                                          | Prospective cohort study | 20 (SCCHN) | Archived tissue and peripheral blood | Evaluate the kinetics of ctDNA in advanced/metastatic SCCHN treated with anti-PD1 agents | - Changes in ctDNA levels in order to correlate with PFS and OS | Recruiting |
| NCT04490564 (CBS-PD-L1a)                                      | Prospective cohort study | 25 | Archived tissue and peripheral blood | Clinical performance of PD-L1 kit in CTCs of peripheral blood and tumor tissue samples. DRFi is defined as the time from the end of primary treatment until the time of diagnosis of a distant recurrence of the Index Cancer. | Correlations between PD-L1 expression in serial liquid samples with patients’ responsiveness to therapy. | Recruiting |
| NCT05059444 (ORACLE)                                          | Prospective cohort study | 1000 (Several cancer types including HNC) | Archived tissue and peripheral blood | Sensitivity, PPV, Lead time defined as the interval between ctDNA detection and clinical detection of recurrence. | - | Recruiting |
| ClinicalTrials.gov (Accessed on 15 May 2022) (Other Study IDs) | Design | N | Sample Type | Primary Endpoint | Secondary Endpoint | Enrollment Status |
|---|---|---|---|---|---|---|
| NCT04599309 (PRE-MERIDAN) | Prospective cohort study | 20 (LA SCCHN candidates for standard definitive therapy) | Archived tissue and serially-collected peripheral blood | Number of high-risk LA-HNSCC patients with successful detection of ctDNA and/or HPV DNA in real time | - Correlation of presence of ctDNA +/- HPV DNA after standard treatment with shorter relapse-free survival (RFS), as assessed by comparison of baseline ctDNA +/- HPV DNA detection with time to relapse - Change in kinetics of ctDNA and/or HPV DNA over time after the end of standard definitive treatment and at recurrence, as assessed by ctDNA/HPV DNA analysis at sequential time points - Selection of the best time-point to detect MRD after standard definitive therapy in SCCHN, as assessed by comparison of quantified ctDNA +/- HPV DNA at 4-6 weeks vs. 8-10 weeks. | Recruiting |
| NCT03712566 (MASST-001) | Prospective cohort study | 39 (Several cancer types including HNC) | Archived tissue and peripheral blood | To comprehensively characterize genomic, epigenetic and immune profiling features and changes in longitudinal blood samples that are associated with systemic treatment of recurrent or metastatic SCCHN. | - Establish a Clinically Annotated Biorepository - Correlate Multi-Omic Results with Clinical Outcome - Compare HPV-Positive and HPV-Negative Cell Histologies - Investigate the Relationship Between Genomic Profiles and Radiomic Signatures | Active, not recruiting |
| NCT05150509 | Prospective cohort study | 110 OSCC | Saliva | To establish a diagnostic test in the early detection of OSCC | - | Recruiting |

cTMDNA: circulating-tumor DNA, DRFI: distant recurrence-free interval, HNC: head and neck cancer, LA: locally advanced, HPV: human papillomavirus, OS: overall survival, OSCC: oral squamous cell carcinoma, PFS: progression-free survival, PPV: positive predictive value, RFS: relapse-free survival, SCCHN: squamous-cell cancer of the head and neck.
4. Conclusions

The liquid biopsy field in head and neck cancer is a growing area of research that has demonstrated the feasibility of detecting CTC, ctDNA, EVs and miRNAs for prognostic and/or predictive purposes. In particular, the detection of ctDNA in virus-associated cancers such as HPV-related OPC and EBV-positive NPC has shown notable prognostic and predictive roles and should be incorporated into the design of future clinical trials. Multigene panel NGS-driven plasma ctDNA detection seems a promising diagnostic tool in HIV-unrelated SCCHN. The different liquid biopsy modalities should be combined with imaging tests in order to maximize their diagnostic accuracy for head and neck cancers.

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References

1. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J. Clin. 2021, 71, 209–249. [CrossRef]
2. Vermorken, J.B.; Remenar, E.; van Herpen, C.; Gorlia, T.; Mesia, R.; Degardin, M.; Stewart, J.S.; Jelic, S.; Betka, J.; Preiss, J.H.; et al. Cisplatin, Fluorouracil, and Docetaxel in Unresectable Head and Neck Cancer. N. Engl. J. Med. 2007, 357, 1695–1704. [CrossRef]
3. Ferris, R.L.; Blumenschein, G., Jr.; Fayette, J.; Colevas, A.D.; Licitra, L.; Harrington, K.; Kasper, S.; Vokes, E.E.; Even, C.; et al. Impact of nivolumab vs. standard, single-agent therapy of investigator’s choice on patient-reported outcomes in recurrent or metastatic squamous cell carcinoma of the head and neck: Health-related quality-of-life results from CheckMate 141, a randomized, phase 3 trial. Lancet Oncol. 2017, 18, 1104–1115. [CrossRef]
4. Kong, L.; Birkeland, A.C. Liquid biopsies in head and neck cancer: Current state and future challenges. Cancers 2021, 13, 1874. [CrossRef]
5. Alix-Panabières, C.; Pantel, K.; Authors, C. Liquid Biopsy: From Discovery to Clinical Application. Cancer Discov. 2021, 11, 858–873. [CrossRef]
6. Wang, Y.; Springer, S.; Mulvey, C.L.; Silliman, N.; Schaefer, J.; James, N.; Rettig, E.M.; Guo, T.; Pickering, C.R.; et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci. Transl. Med. 2015, 7, 293ra104. [CrossRef]
7. Stransky, N.; Egloff, A.M.; Tward, A.D.; Costic, A.D.; Cibulskis, K.; Sikachenko, A.; Kryukov, G.V.; Lawrence, M.; Sougnez, C.; Mckenna, A.; et al. The mutational landscape of head and neck squamous cell carcinoma. Science 2011, 333, 1157–1160. [CrossRef]
8. Lawrence, M.S.; Sougnez, C.; Lichtenstein, L.; Cibulskis, K.; Lander, E.; Gabriel, S.B.; Getz, G.; Ally, A.; Balasundaram, M.; Biro, I.; et al. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 2015, 517, 576–582. [CrossRef]
9. Whiteside, T.L. Head and neck carcinoma immunotherapy: Facts and hopes. Clin. Cancer Res. 2018, 24, 6–13. [CrossRef]
10. Theodoraki, M.N.; Yerneni, S.; Gooding, W.E.; Ohr, J.; Clump, D.A.; Bauman, J.E.; Ferris, R.L.; Whiteside, T.L. Circulating exosomes measure responses to therapy in head and neck cancer patients treated with cetuximab, ipilimumab, and IMRT. Oncoimmunology 2019, 8, e1593805. [CrossRef]
11. Lin, J.C.; Tsai, C.S.; Wang, W.Y.; Jan, J.S. Detection of Circulating Tumor Cells in Venous Blood of Nasopharyngeal Carcinoma Patients by Nested Reverse Transcriptase-Polymerase Chain Reaction. Kaohsiung J. Med. Sci. 2000, 16, 1–8.
12. Nakashima, H.; Yoshida, R.; Hirose, A.; Kawahara, K.; Sakata, J.; Arita, H.; Yamamoto, T.; Toya, R.; Murakami, R.; Hiraki, A.; et al. Circulating miRNA-1290 as a potential biomarker for response to chemoradiotherapy and prognosis of patients with advanced oral squamous cell carcinoma: A single-center retrospective study. Tumor Biol. 2019, 41, 1–10. [CrossRef]
13. Zhang, G.; Zong, J.; Lin, S.; Verhoeven, R.J.A.; Tong, S.; Chen, Y.; Ji, M.; Cheng, W.; Tsao, S.W.; Lung, M.; et al. Circulating Epstein-Barr virus microRNAs miR-BART7 and miR-BART13 as biomarkers for nasopharyngeal carcinoma diagnosis and treatment. Int. J. Cancer 2015, 136, E301–E312. [CrossRef]
14. Ramayanti, O.; Verkuilen, S.A.W.M.; Novianti, P.; Scheepbouwer, C.; Misovic, B.; Koppers-Lalic, D.; van Weering, J.; Beckers, L.; Adham, M.; Martorelli, D.; et al. Vesicle-bound EBV-BART13-3p miRNA in circulation distinguishes nasopharyngeal from other head and neck cancer and asymptomatic EBV-infections. *Int. J. Cancer* 2019, 144, 2555–2566. [CrossRef]

15. Onidani, K.; Shoji, H.; Kakizaki, T.; Yoshimoto, S.; Okaya, S.; Miura, N.; Sekikawa, S.; Furuta, K.; Lim, C.T.; Shibahara, T.; et al. Monitoring of cancer patients via next-generation sequencing of patient-derived circulating tumor cells and tumor DNA. *Cancer Sci.* 2019, 110, 2590–2599. [CrossRef]

16. Wirtschafter, A.; Benninger, M.S.; Moss, T.J.; Umiel, T.; Blazoff, K.; Worsham, M.J. Micrometastatic tumor detection in patients with head and neck cancer: A preliminary report. *Arch. Otolaryngol.—Head Neck Surg.* 2002, 128, 40–43. [CrossRef]

17. Partridge, M.; Brakenhoff, R.; Phillips, E.; Ali, K.; Francis, R.; Hooper, R.; Lavery, K.; Brown, A.; Langdon, J. Detection of Rare Disseminated Tumor Cells Identifies Head and Neck Cancer Patients at Risk of Treatment Failure. *Clin. Cancer Res.* 2003, 9, 5287–5294.

18. Guney, K.; Yoldas, B.; Ozbilim, G.; Derin, A.T.; Sarihan, S.; Balkan, E. Detection of micrometastatic tumor cells in head and neck squamous cell carcinoma. A possible predictor of recurrences? *Saudi Med. J.* 2007, 28, 216–220.

19. Winter, S.C.; Stephenson, S.A.; Subramaniam, S.K.; Paleri, V.; Ha, K.; Marnane, C.; Krishnan, S.; Rees, G. Long term survival following the detection of circulating tumour cells in head and neck squamous cell carcinoma. *BMC Cancer* 2009, 9, 424. [CrossRef]

20. Gdoutos, E.E.; Balasubramanian, P.; Lang, J.C.; Yang, L.; Jatana, C.A.; White, E.; Agrawal, A.; Ozer, E.; Schuller, D.E.; Teknos, T.N.; et al. Significance of circulating tumor cells in patients with squamous cell carcinoma of the head and neck: Initial results. *Arch. Otolaryngol.—Head Neck Surg.* 2010, 136, 1274–1279. [CrossRef]

21. Buglione, M.; Grisanti, S.; Almici, C.; Mangoni, M.; Polli, C.; Consoli, F.; Verardi, R.; Costa, L.; Paiar, F.; Pasinetti, N.; et al. Circulating tumour cells in locally advanced head and neck cancer: Preliminary report about their possible role in predicting response to non-surgical treatment and survival. *Eur. J. Cancer* 2012, 48, 3019–3026. [CrossRef]

22. Nichols, A.C.; Lowes, L.E.; Szeto, C.C.; Basmaji, J.; Dhaliwal, S.; Chapeskie, C.; Todorovic, B.; Read, N.; Venkatesan, V.; Hammond, A.; et al. Detection of circulating tumor cells in advanced head and neck cancer using the CellSearch system. *Head Neck* 2012, 34, 1440–1444. [CrossRef]

23. Bozec, A.; Ilié, M.; Dassonville, O.; Long, E.; Poissonnet, G.; Santini, J.; Chamorey, E.; Ettaiche, M.; Chauvière, D.; Peyrade, F.; et al. Significance of circulating tumor cell detection using the CellSearch system in patients with locally advanced head and neck squamous cell carcinoma. *Eur. Arch. Oto-Rhino-Laryngol.* 2013, 270, 2745–2749. [CrossRef]

24. He, S.; Li, P.; Long, T.; Zhang, N.; Yu, Z. Detection of circulating tumour cells with the CellSearch system in patients with advanced-stage head and neck cancer: Preliminary results. *J. Laryngol. Otol.* 2013, 127, 788–793. [CrossRef]

25. Hsieh, J.C.; Lin, H.C.; Huang, C.Y.; Hsu, H.L.; Wu, T.M.; Lee, C.L.; Chen, M.C.; Wang, H.M.; Tseng, C. Prognostic value of circulating tumor cells with podoplanin expression in patients with locally advanced or metastatic head and neck squamous cell carcinoma. *Head Neck* 2015, 37, 1448–1455. [CrossRef]

26. Hristozova, T.; Konschak, R.; Stromberger, C.; Fusi, A.; Liu, Z.; Weichert, W.; Stenzinger, A.; Budach, V.; Tinhofer, I. The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN). *Ann. Oncol.* 2011, 22, 1878–1885. [CrossRef]

27. Tinhofer, I.; Konschak, R.; Stromberger, C.; Raguse, J.D.; Dreyer, J.H.; Jöhrens, K.; Keilholz, U.; Budach, V. Detection of circulating tumor cells for prediction of recurrence after adjuvant chemoradiation in locally advanced squamous cell carcinoma of the head and neck. *Ann. Oncol.* 2014, 25, 2042–2047. [CrossRef]

28. Grisanti, S.; Almici, C.; Consoli, F.; Buglione, M.; Verardi, R.; Bolzoni-Villaret, A.; Bianchetti, A.; Ciccarese, C.; Mangoni, M.; Ferrari, L.; et al. Circulating tumor cells in patients with recurrent or metastatic head and neck carcinoma: Prognostic and predictive significance. *PloS ONE* 2014, 9, e103918. [CrossRef]

29. Inhesterin, J.; Oertel, K.; Stemmann, V.; Schmalenberg, H.; Dietz, A.; Rotter, N.; Veit, J.; Görner, M.; Sudhoff, H.; Junghansb, C.; et al. Prognostic role of circulating tumor cells during induction chemotherapy followed by curative surgery combined with postoperative radiotherapy in patients with locally advanced oral and oropharyngeal squamous cell cancer. *PloS ONE* 2015, 10, e0132901. [CrossRef]

30. Kusukawa, J.; Suetofuji, Y.; Ryu, F.; Noguchi, R.; Iwamoto, O.; Kameyama, T. Dissemination of cancer cells into circulation occurs by incisional biopsy of oral squamous cell carcinoma. *J. Oral Pathol. Med.* 2000, 29, 303–307. [CrossRef]

31. Garrel, R.; Mazel, M.; Perriard, F.; Vinches, M.; Cayrefourcq, L.; Guigay, J.; Digue, L.; Aubry, K.; Alfonso, M.; Delord, J.P.; et al. Circulating Tumor Cells as a Prognostic Factor in Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma: The CIRCUTEC Prospective Study. *Clin. Chem.* 2019, 65, 1267–1275. [CrossRef] [PubMed]

32. Chikamatsu, K.; Tada, H.; Takahashi, H.; Kuwabara-Yokobori, Y.; Ishii, H.; Ida, S.; Shino, M. Expression of immune-regulatory molecules in circulating tumor cells derived from patients with head and neck squamous cell carcinoma. *Oral Oncol.* 2019, 89, 34–39. [CrossRef] [PubMed]

33. Kulasinghe, A.; Perry, C.; Kennedy, L.; Warkiani, M.E.; Nelson, C.; Punyadeera, C. PD-L1 expressing circulating tumour cells in head and neck cancers. *BMC Cancer* 2017, 17, 333. [CrossRef] [PubMed]

34. Gröbe, A.; Blessmann, M.; Hanken, H.; Friedrich, R.E.; Schön, G.; Wikner, J.; Effenberger, K.E.; Kluwe, L.; Heiland, M.; Pantel, K.; et al. Prognostic relevance of circulating tumor cells in blood and disseminated tumor cells in bone marrow of patients with squamous cell carcinoma of the oral cavity. *Clin. Cancer Res.* 2014, 20, 425–433. [CrossRef] [PubMed]
35. Strati, A.; Koutsodontis, G.; Papaxonis, G.; Angelidis, I.; Zavridou, M.; Economopoulou, P.; Kotsantis, I.; Avgeris, M.; Mazel, M.; Perisanidis, C.; et al. Prognostic significance of PD-L1 expression on circulating tumor cells in patients with head and neck squamous cell carcinoma. *Ann. Oncol.* 2017, 28, 1923–1933. [CrossRef]

36. Kulasinghe, A.; Kenny, L.; Perry, C.; Thiery, J.P.; Jovanovic, I.; Vela, I.; Nelson, C.; Punyadeera, C. Impact of label-free technologies in head and neck cancer circulating tumour cells. *Oncotarget* 2016, 7, 71223–71234. [CrossRef]

37. Dyavanagoudar, S.; Kale, A.; Bhat, K.; Hallikerimath, S. Reverse transcriptase polymerase chain reaction study to evaluate dissemination of cancer cells into circulation after incision biopsy in oral squamous cell carcinoma. *Indian J. Dent. Res.* 2008, 19, 315–319. [CrossRef]

38. Liao, C.-J.; Hsieh, C.-H.; Hung, F.-C.; Wang, H.-M.; Chou, W.-P.; Wu, M.-H. The Integration of a Three-Dimensional Spheroid Cell Culture Operation in a Circulating Tumor Cell (CTC) Isolation and Purification Process: A Preliminary Study of the Clinical Significance and Prognostic Role of the CTCs Isolated from the Blood Samples of Head and Neck Cancer Patients. *Cancers* 2019, 11, 783. [CrossRef]

39. Zheng, W.; Zhang, Y.; Guo, L.; Wang, S.; Fang, M.; Mao, W.; Lou, J. Evaluation of therapeutic efficacy with CytoSorter® circulating tumor cell-capture system in patients with locally advanced head and neck squamous cell carcinoma. *Cancer Manag. Res.* 2019, 11, 5857–5869. [CrossRef]

40. Chang, P.H.; Wu, M.H.; Liu, S.Y.; Wang, H.M.; Huang, W.K.; Liao, C.T.; Yen, T.C.; Ng, S.H.; Chen, J.S.; Lin, Y.C.; et al. The prognostic roles of pretreatment circulating tumor cells, circulating cancer stem-like cells, and programmed cell death-1 expression on peripheral lymphocytes in patients with initially unresectable, recurrent or metastatic head and neck cancer: An Exploratory Study of Three Biomarkers in One-time Blood Drawing. *Cancers* 2019, 11, 540. [CrossRef]

41. Wang, H.M.; Wu, M.H.; Chang, P.H.; Lin, H.C.; Liao, C.D.; Wu, S.M.; Hung, T.M.; Lin, C.Y.; Chang, T.C.; Tzu-Tsen, Y.; et al. The change in circulating tumor cells before and during concurrent chemoradiation is associated with survival in patients with locally advanced head and neck cancer. *Head Neck* 2019, 41, 2676–2687. [CrossRef] [PubMed]

42. Kawada, T.; Takahashi, H.; Sakakura, K.; Ida, S.; Mito, I.; Toyoda, M.; Chikamatsu, K. Circulating tumor cells in patients with head and neck squamous cell carcinoma: Feasibility of detection and quantitation. *Head Neck* 2017, 39, 2180–2186. [CrossRef] [PubMed]

43. Morgan, T.M.; Wang, X.; Qian, X.; Switchenko, J.M.; Nie, S.; Patel, K.R.; Cassidy, R.J.; Shin, D.M.; Beittler, J.J. Measurement of circulating tumor cells in squamous cell carcinoma of the head and neck and patient outcomes. *Clin. Transl. Oncol.* 2019, 21, 342–347. [CrossRef] [PubMed]

44. Sun, T.; Zou, K.; Yuan, Z.; Yang, C.; Lin, X.; Xiong, B. Clinicopathological and prognostic significance of circulating tumor cells in squamous cell carcinoma of the head and neck. *Oncol. Lett.* 2016, 11, 5857–5869. [CrossRef] [PubMed]

45. Ng, S.P.; Bahig, H.; Wang, J.; Cardenas, C.E.; Lucci, A.; Hall, C.S.; Meas, S.; Sarli, V.N.; Yuan, Y.; Urbauer, D.L.; et al. Predicting treatment Response based on Dual assessment of magnetic resonance Imaging kinetics and Circulating Tumor cells in patients with Head and Neck cancer (PREDICT-HN): Matching “liquid biopsy” and quantitative tumor modeling. *BMC Cancer* 2018, 18, 903. [CrossRef] [PubMed]

46. Zhang, J.; Shi, H.; Jiang, T.; Liu, Z.; Lin, P.P.; Chen, N. circulating tumor cells with karyotyping as a novel biomarker for diagnosis and treatment of nasopharyngeal carcinoma. *BMC Cancer* 2018, 18, 1133. [CrossRef]

47. Si, Y.; Lan, G.; Deng, Z.; Wang, Y.; Lu, Y.; Qin, Y.; Huang, B.; Yang, Y.; Weng, J.; Han, X.; et al. Distribution and clinical significance of circulating tumor cells in nasopharyngeal carcinoma. *Ipm. J. Clin. Oncol.* 2016, 46, 622–630. [CrossRef]

48. You, R.; Liu, Y.P.; Lin, M.; Huang, P.Y.; Tang, L.Q.; Zhang, Y.N.; Pan, Y.; Liu, W.L.; Guo, W.B.; Zou, X.; et al. Relationship of circulating tumor cells and Epstein-Barr virus DNA to progression-free survival and overall survival in metastatic nasopharyngeal carcinoma patients. *Int. J. Cancer* 2019, 145, 2873–2883. [CrossRef]

49. Li, Y.J.; Luo, Y.; Xie, X.Q.; Li, P.; Wang, F. The prognostic value of COX-2 expression on circulating tumor cells in patients with esophageal cancer: A meta-analysis. *Oncotargets Ther.* 2017, 10, 3907–3916. [CrossRef]

50. Vo, J.H.; Nei, W.L.; Hu, M.; Phyo, W.M.; Wang, F.; Fong, K.W.; Tan, T.; Soong, Y.L.; Cheah, S.L.; Sommat, K.; et al. Comparison of Circulating Tumour Cells and Circulating Cell-Free Epstein-Barr Virus DNA in Patients with Nasopharyngeal Carcinoma Undergoing Radiotherapy. *Sci. Rep.* 2016, 6, 13. [CrossRef]

51. He, C.; Huang, X.; Su, X.; Tang, T.; Zhang, X.; Ma, J.; Guo, X.; Lv, X. The association between circulating tumor cells and Epstein-Barr virus activation in patients with nasopharyngeal carcinoma. *Cancer Biol. Ther.* 2017, 18, 888–894. [CrossRef] [PubMed]

52. Fu, X.; Shen, C.; Wang, H.; Chen, F.; Li, G.; Wen, Z. Joint quantitative measurement of hTERT mRNA in both peripheral blood and circulating tumor cells of patients with nasopharyngeal carcinoma and its clinical significance. *BMC Cancer* 2017, 17, 4–11. [CrossRef] [PubMed]

53. Ou, G.; Xing, S.; Li, J.; Zhang, L.; Chen, S. Circulating tumor cells: A valuable marker of poor prognosis for advanced nasopharyngeal carcinoma. *Mol. Med.* 2019, 25, 50. [CrossRef]

54. Sun, L.; Wang, Y.; Shi, J.; Zhu, W.; Wang, X. Association of Plasma Epstein-Barr Virus LMP1 and EBER1 with Circulating Tumor Cells and the Metastasis of Nasopharyngeal Carcinoma. *Pathol. Oncol. Res.* 2020, 26, 1893–1901. [CrossRef]

55. Wen, Z.; Li, Z.; Yong, P.; Liang, D.; Xie, D.; Chen, H.; Yang, Y.; Wu, S.; Li, C.; Cheng, Z. Detection and clinical significance of circulating tumor cells in patients with nasopharyngeal carcinoma. *Oncof. Lett.* 2019, 18, 2537–2547. [CrossRef] [PubMed]
56. Xie, X.; Luo, Y.; Ma, X.L.; Li, S.; Liu, L.; Zhang, H.; Li, P.; Wang, F. Clinical significance of circulating tumor cells and their expression of cyclooxygenase-2 in patients with nasopharyngeal carcinoma. *Eur. Rev. Med. Pharmacol. Sci.* 2019, 23, 6951–6961. [CrossRef]

57. Seethala, R.R.; Stemen, G. Update from the 4th Edition of the World Health Organization Classification of Head and Neck Tumors: Tumors of the Salivary Gland. *Head Neck Pathol.* 2017, 11, 55–67. [CrossRef]

58. Kim, Y.; Song, S.; Lee, M.; Swatloski, T.; Kang, J.H.; Ko, Y.H.; Park, W.Y.; Jeong, H.S.; Park, K. Integrative genomic analysis of salivary duct carcinoma. *Sci. Rep.* 2020, 10, 14995. [CrossRef]

59. Schmitt, N.C.; Kang, H.; Sharma, A. Salivary Duct Carcinoma: An aggressive Salivary Gland Malignancy with Opportunities for Targeted Therapy. *Oral Oncol.* 2017, 74, 40–48. [CrossRef]

60. Asai, S.; Sumiyoshi, S.; Yamada, Y.; Tateya, I.; Nagao, T.; Minamiguchi, S.; Haga, H. High-grade salivary gland carcinoma with the ETV6-NTRK3 gene fusion: A case report and literature review of secretory carcinoma with high-grade transformation. *Pathol. Int.* 2021, 71, 427–434. [CrossRef]

61. Fisher, B.M.; Tang, K.D.; Warkiani, M.E.; Punyadeera, C.; Batstone, M.D. A pilot study for presence of circulating tumour cells in adenoid cystic carcinoma. *Int. J. Oral Maxillofac. Surg.* 2021, 50, 994–998. [CrossRef] [PubMed]

62. Cappelletti, V.; Miodini, P.; Reduzzi, C.; Alfieri, S.; Daidone, M.G.; Licitra, L.; Locati, L.D. Tailoring treatment of salivary duct carcinoma (SDC) by liquid biopsy: ARV7 expression in circulating tumour cells. *Ann. Oncol.* 2018, 29, 1598–1600. [CrossRef] [PubMed]

63. CabEZas-Camarero, S.; de la Orden García, V.; García-Barberán, V.; Mediero-Valeros, B.; Subhi-Issa, A.L.; Llovet García, P.; Bando-Polaino, I.; Merino-Menéndez, S.; Pérez-Segura, P.; Díaz-Rubio, E. Nasoethmoidal Intestinal-Type Adenocarcinoma Treated with Cetuximab: Role of Liquid Biopsy and BEAMing in Predicting Response to Anti-Epidermal Growth Factor Receptor Therapy. *Oncologist* 2019, 24, 293–300. [CrossRef] [PubMed]

64. Lin, L.H.; Chang, K.W.; Kao, S.Y.; Cheng, H.W.; Liu, C.J. Increased plasma circulating cell-free DNA could be a potential marker for oral cancer. *Int. J. Mol. Sci.* 2019, 19, 3303. [CrossRef] [PubMed]

65. Perdomo, S.; Avogbe, P.H.; Foll, M.; Abedi-Ardekani, B.; Fasciola, V.L.; Anantharaman, D.; Chopard, P.; Le Calvez-Kelm, F.; Vilensky, M.; Polesel, J.; et al. Circulating tumor DNA detection in head and neck cancer: Evaluation of two different detection approaches. *OncoTarget* 2017, 8, 72621–72632. [CrossRef]

66. Fostira, F.; Oikonomopoulou, P.; Kladí, A.; Edelstein, D.; Stieler, K.; Heim, D.; Gkotzamanidou, M.; Anastasiou, M.; Kotsantis, I.; Kavourakis, E.; et al. Blood-based testing of mutations in patients with head and neck squamous cell carcinoma (HNSCC) using highly sensitive SafeSeq technology. *Ann. Oncol.* 2019, 30 (Suppl. S5), v449–v474. [CrossRef]

67. Flach, S.; Howarth, K.; Hackinger, S.; Pipinikas, C.; Ellis, P.; Mc Kay, L.; Marsico, G.; Forshew, T.; Walz, C.; Reichel, C.A.; et al. Liquid Biopsy for MiNimal RESidual DiSease Detection in Head and Neck Squamous Cell Carcinoma (LIONESS)—A personalised circulating tumour DNA analysis in head and neck squamous cell carcinoma. *Br. J. Cancer* 2022, 126, 1186–1195. [CrossRef]

68. Li, W.; Wildsmith, S.; Ye, J.; Han, S.; Morsli, N.; He, P.; Shetty, J.; Yovine, A.J.; Holoweckyj, N.; Raja, R.; et al. Plasma-based tumor mutational burden (bTMB) as predictor for survival in phase III EAGLE study: Durvalumab (D) ± tremelimumab (T) versus chemotherapy (CT) in recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) after platinum failure. *ASCO Virtual Meeting 2020. J. Clin. Oncol.* 2020, 38 (Suppl. S15), e1499–e1500. [CrossRef] [PubMed]

69. Porter, A.; Natsuhara, M.; Daniels, G.A.; Patel, S.P.; Sacco, A.G.; Bykowski, J.; Banks, K.C.; Cohen, E.E. Next generation sequencing approaches. *Acta Oncol.* 2018, 57, 4682–4690. [CrossRef] [PubMed]

70. Wilson, H.L.; D’Agostino, R.B., Jr.; Meegalla, N.; Petro, R.; Commander, S.; Topaloglu, U.; Zhang, W.; Porosnicu, M. The prognostic and therapeutic value of the mutational profile of blood and tumor tissue in head and neck squamous cell carcinoma. *Clin. Cancer Res.* 2021, 27, 2023–2030. [CrossRef] [PubMed]

71. Van Ginkel, J.H.; Huibers, M.M.H.; van Es, R.J.J.; de Bree, R.; Willems, S.M. Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. *BMC Cancer* 2017, 17, 428. [CrossRef] [PubMed]

72. Egyud, M.; Sridhar, P.; Devaiah, A.; Yamada, E.; Saunders, S.; Stahlberg, A.; Filges, S.; Krzyzanowski, P.M.; Kalatskaya, I.; Jiao, W.; et al. Plasma circulating tumor DNA as a potential tool for disease monitoring in head and neck cancer. *Head Neck Cancer* 2019, 41, 1351–1358. [CrossRef] [PubMed]

73. Pall, A.H.; Jakobsen, K.K.; Gronhøj, C.; von Buchwald, C. Circulating tumour DNA alterations as biomarkers for head and neck cancer: A systematic review. *Acta Oncol.* 2020, 59, 845–850. [CrossRef]

74. Hudecková, M.; Kouchy, V.; Rottenberg, J.; Gál, B. Gene Mutations in Circulating Tumour DNA as a Diagnostic and Prognostic Marker in Head and Neck Cancer—A Systematic Review. *BioMedicines* 2021, 9, 1548. [CrossRef] [PubMed]

75. Damerla, R.R.; Lee, N.Y.; You, D.; Soni, R.; Shah, R.; Reynoldg, M.; Katabi, N.; Wu, V.; McBride, S.M.; Tsai, C.J.; et al. Detection of Early Human Papillomavirus–Associated Cancers by Liquid Biopsy. *JCO Precis. Oncol.* 2019, 3, 1–17. [CrossRef] [PubMed]

76. Chera, B.S.; Kumar, S.; Beatty, B.T.; Marron, D.; Jeffersy, S.; Green, R.; Goldman, E.C.; Amdur, R.; Sheets, N.; Dagan, R.; et al. Rapid Clearance Profile of Plasma Circulating Tumor HPV Type 16 DNA During Chemoradiotherapy Correlates With Disease Control in HPV-Associated Oropharyngeal Cancer. *Clin. Cancer Res.* 2019, 25, 4682–4690. [CrossRef] [PubMed]

77. Dahlstrom, K.R.; Li, G.; Hussey, C.S.; Vo, J.T.; Wei, Q.; Zhao, C.; Sturgis, E.M. Circulating human papillomavirus DNA as a marker for disease extent and recurrence among patients with oropharyngeal cancer. *Cancer* 2015, 121, 3455–3464. [CrossRef]
83. Akashi, K.; Sakai, T.; Fukuoka, O.; Saito, Y.; Yoshida, M.; Ando, M.; Ito, T.; Murakami, Y.; Yamasoba, T. Usefulness of circulating DNA Load After Induction Chemotherapy Predicts Outcome in Locoregionally Advanced Nasopharyngeal Carcinoma. *Int. J. Radiat. Oncol. Biol. Phys.* 2012, 82, e351–e358. [CrossRef]

84. Chen, Q.-Y.; Guo, S.-Y.; Tang, L.-Q.; Lu, T.-Y.; Chen, B.-L.; Zhong, Q.-Y.; Zhong, P.-L.; Tang, Q.-N.; Chen, W.-H.; Guo, S.S.; et al. Combination of Tumor Volume and Epstein-Barr Virus DNA Improved Prognostic Stratification of Stage II Nasopharyngeal Carcinoma in the Immune-Modulated Radiotherapy Era: A Large-Scale Cohort Study. *Cancer Res. Treat.* 2019, 50, 861–871. [CrossRef] [PubMed]

85. Guo, R.; Tang, L.L.; Mao, Y.P.; Du, X.; Shen, L.; Zhang, Z.C.; Liu, L.-Z.; Tian, L.; Luo, X.-T.; Xie, Y.B.; et al. Proposed modifications and incorporation of plasma Epstein-Barr virus DNA improve the TNM staging system for Epstein-Barr virus-related nasopharyngeal carcinoma. *Cancer* 2019, 125, 79–89. [CrossRef]

86. Huang, C.L.; Sun, Z.Q.; Guo, R.; Liu, X.; Mao, Y.P.; Peng, H.; Tian, L.; Lin, A.-H.; Li, L.; Shao, J.-Y.; et al. Plasma Epstein-Barr Virus DNA Load After Induction Chemotherapy Predicts Outcome in Locoregionally Advanced Nasopharyngeal Carcinoma. *Int. J. Radiat. Oncol. Biol. Phys.* 2019, 104, 355–361. [CrossRef]

87. Liu, L.T.; Tang, L.Q.; Chen, Q.Y.; Lu, Z.; Guo, S.S.; Guo, L.; Mo, H.-Y.; Zhao, C.; Guo, X.; Cao, K.-J.; et al. The addition of pretreatment plasma Epstein–Barr virus DNA into the eighth edition of nasopharyngeal cancer TNM stage classification. *Int. J. Cancer* 2019, 144, 1713–1722. [CrossRef] [PubMed]

88. Xu, C.; Zhang, S.; Li, W.F.; Chen, L.; Mao, Y.P.; Guo, Y.; Liu, Q.; Ma, J.; Tang, L.-L. Selection and Validation of Induction Chemotherapy Beneficiaries Among Patients With T3N0, T3N1, T4N0 Nasopharyngeal Carcinoma Using Epstein-Barr Virus DNA: A Joint Analysis of Real-World and Clinical Trial Data. *Front. Oncol.* 2019, 9, 1343. [CrossRef]

89. Zhang, L.; Tang, L.Q.; Liu, T.-Y.; Chen, B.-L.; Zhong, Q.-Y.; Zou, M.-S.; Tang, Q.-N.; Chen, W.-H.; Guo, S.S.; et al. Analysis of plasma Epstein-Barr viral DNA to screen for nasopharyngeal cancer. *Oncotarget* 2016, 7, 6221–6230. [CrossRef] [PubMed]

90. Liu, L.T.; Tang, L.Q.; Chen, Q.Y.; Lu, H.; Guo, S.-S.; Liu, L.-T.; Guo, L.; Mo, H.-Y.; Zhao, C.; Guo, X.; Cao, K.-J.; et al. Cell-free papillomavirus DNA kinetics after surgery for human papillomavirus-associated oropharyngeal cancer. *Cancer* 2012, 128, 2193–2204. [CrossRef]

91. Chen, Q.-Y.; Guo, S.-Y.; Tang, L.-Q.; Lu, T.-Y.; Chen, B.-L.; Zhong, Q.-Y.; Zou, M.-S.; Tang, Q.-N.; Chen, W.-H.; Guo, S.S.; et al. Usefulness of circulating tumor DNA by targeting human papilloma virus-derived sequences as a biomarker in p16-positive oropharyngeal cancer. *Sci. Rep.* 2012, 2, 572. [CrossRef] [PubMed]

92. Lee, V.H.F.; Kwong, D.L.W.; Leung, T.W.; Choi, C.W.; O’Sullivan, B.; Lam, K.O.; Lai, V.; Khong, P.L.; Chan, S.K.; Ng, C.Y.; et al. The addition of pretreatment plasma Epstein–Barr virus DNA into the eighth edition of nasopharyngeal cancer TNM stage classification. *Int. J. Cancer* 2019, 144, 1713–1722. [CrossRef] [PubMed]

93. Mazurek, A.M.; Rutkowski, T.; Fiszer-Kierzkowska, A.; Mahusecka, E.; Składowski, K. Assessment of the total cfDNA and quantative polymerase chain reaction-based detection and surveillance of human papillomavirus-related head and neck cancer. *JAMA Otolaryngol.—Head Neck Surg.* 2014, 140, 846–854. [CrossRef]

94. Cabezas-Camarero, S.; Pérez-Alfayate, R.; Puebla, F.; Cabrera-Martín, M.N.; Pérez-Segura, P. Increased clinical and plasma EBV DNA responses to platinum-gemcitabine after nivolumab in patients with heavily platinum-pretreated nasopharyngeal carcinoma. *Oral Oncol.* 2020, 103, 104527. [CrossRef] [PubMed]

95. Chan, K.C.A.; Woo, J.K.S.; King, A.; Zee, B.C.Y.; Lam, W.K.J.; Chan, S.L.; Chu, S.W.I.; Mak, C.; Tse, I.O.L.; Leung, S.Y.M.; et al. Analysis of plasma Epstein–Barr virus DNA to screen for nasopharyngeal cancer. *N. Engl. J. Med.* 2017, 377, 513–522. [CrossRef] [PubMed]

96. Theodoraki, M.N.; Yerneni, S.S.; Brunner, C.; Theodorakis, J.; Hoffmann, T.K.; Whiteside, T.L. Plasma-derived exosomes reverse epithelial-to-mesenchymal transition after photodynamic therapy of patients with head and neck cancer. *OncoSience* 2018, 5, 75–87. [CrossRef]

97. Wang, J.; Zhou, Y.; Lu, J.; Sun, Y.; Xiao, H.; Liu, M.; Tian, L. Combined detection of serum exosomal miR-21 and HOTAIR as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma. *Med. Oncol.* 2014, 31, 148. [CrossRef]
98. Kang, J.W.; Eun, Y.G.; Lee, Y.C. Diagnostic value of salivary miRNA in head and neck squamous cell cancer: Systematic review and meta-analysis. *Int. J. Mol. Sci.* 2021, 22, 7026. [CrossRef]

99. Rapado-González, O.; Martínez-Reglero, C.; Salgado-Barreira, A.; López-López, R.; Suárez-Cunqueiro, M.M.; Muinelo-Romay, L. miRNAs in liquid biopsy for oral squamous cell carcinoma diagnosis: Systematic review and meta-analysis. *Oral Oncol.* 2019, 99, 104465. [CrossRef]

100. Lu, Y.C.; Chang, J.T.C.; Huang, Y.C.; Huang, C.C.; Chen, W.H.; Lee, L.Y.; Huang, B.-S.; Chen, Y.-J.; Li, H.-F.; Cheng, A.-J. Combined determination of circulating miR-196a and miR-196b levels produces high sensitivity and specificity for early detection of oral cancer. *Clin. Biochem.* 2015, 48, 115–121. [CrossRef]

101. Liu, C.J.; Lin, S.C.; Yang, C.C.; Cheng, H.W.; Chang, K.W. Exploiting salivary miR-31 as a clinical biomarker of oral squamous cell carcinoma. *Head Neck* 2012, 34, 219–224. [CrossRef] [PubMed]

102. Fayda, M.; Isin, M.; Tambas, M.; Guveli, M.; Meral, R.; Altun, M.; Sahin, D.; Ozkan, G.; Sanli, Y.; Isin, H.; et al. Do circulating long non-coding RNAs (lncRNAs) (lincRNA-p21, GAS 5, HOATIR) predict the treatment response in patients with head and neck cancer treated with chemoradiotherapy? *Tumor Biol.* 2016, 37, 3969–3978. [CrossRef] [PubMed]

103. Lubov, J.; Maschietto, M.; Ibrahim, I.; Mlynarek, A.; Hier, M.; Kowalski, L.P.; Alouai-Jamali, M.A.; da Silva, S.D. Meta-analysis of microRNAs expression in head and neck cancer: Uncovering association with outcome and mechanisms. *Oncotarget* 2017, 8, 55511–55524. [CrossRef] [PubMed]

104. Lousada-Fernandez, F.; Rapado-Gonzalez, O.; Lopez-Cedrun, J.L.; Lopez-Lopez, R.; Muinelo-Romay, L.; Suarez-Cunqueiro, M.M. Liquid biopsy in oral cancer. *Int. J. Mol. Sci.* 2018, 19, 1704. [CrossRef]

105. Adeola, H.A.; Bello, I.O.; Aruleba, R.T.; Francisco, N.M.; Adekiya, T.A.; Adefuye, A.O.; Ikwegbue, P.C.; Musaigwa, F. The Practicality of the Use of Liquid Biopsy in Early Diagnosis and Treatment Monitoring of Oral Cancer in Resource-Limited Settings. *Cancers* 2022, 14, 1139. [CrossRef]