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Hot Topic

Current applications and challenges of circulating tumor DNA (ctDNA) in squamous cell carcinoma of the head and neck (SCCHN)

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ABSTRACT

Liquid biopsies (LB) are emerging in the oncology field, with promising data as new diagnostic, prognostic and treatment-monitoring tools. Squamous cell carcinoma of the head and neck (SCCHN) is a heterogenous disease and many challenges remain to improve patient outcomes. Liquid biopsy could be of interest at different stages of SCCHN disease, including better screening to diagnose more patients at an early stage, early detection of relapse after curative treatment, and the implementation of precision medicine. As LB is very attractive by the ease of sampling, this field is moving fast. Therefore, it is important to be aware of the potential applications but also the limitations of these new tools in regards to technical aspects and interpretation of the data. In this review, we will first give an overview of potential clinical applications and technical challenges of circulating tumor DNA (ctDNA) and then focus on current available data of ctDNA in SCCHN. Although the literature on ctDNA analysis for SCCHN is scarce compared to other tumors, preliminary results seem to hold promise for the future, including the detection of minimal residual disease or the detection of potentially targetable events through liquid biopsy. Prospective liquid-biopsy driven clinical trials are needed to validate its clinical relevance.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the seventh most common malignancy and is responsible for 3.7% of cancer deaths worldwide [1]. The main risk factors are alcohol and tobacco. The human papillomavirus (HPV) is another risk factor for oropharyngeal cancer (OPC) and its incidence is rising [2].The 5-year survival rate of SCCHN is still poor, ranging from 25% to 60% depending on tumor staging and site [3]. Most patients present with locally-advanced disease, but despite multimodal treatment including chemoradiation or surgery followed by (chemo)radiation, > 50% of patients will relapse. Once patients have incurable recurrent/metastatic (R/M) disease, the standard of care is palliative systemic treatment. Platinum-based chemotherapy, programmed cell-death 1 (PD-1) inhibitors and cetuximab improve survival, but the prognosis is dismal with an overall survival of 10–15 months [4,5]. Many challenges remain to improve patient outcomes in SCCHN including better screening to diagnose more patients at an early stage, early detection of relapse after curative treatment, and the implementation of precision medicine.

Liquid biopsies (LB) are emerging in the oncology field with promising data as new diagnostic, prognostic and treatment-monitoring tools [6–8]. LB refers to the sampling and analysis of several biological fluids as opposed to solid biopsy which analyzes a tissue sample [9]. The most popular form of LB is the analysis of blood samples, but LB can also refer to samples of saliva, urine, ascites, cerebrospinal fluids and pleural effusions. Different composites are exploitable in a blood-based biopsy to characterize tumors, including circulating tumor cells (CTCs) [10], circulating tumor DNA (ctDNA), circulating cell-free RNA, circulating extracellular vesicles (EVs), proteins and metabolites. Besides genomics, including the mutational profile and copy number variations, LB can provide information regarding transcriptomics [11], epigenomics [12], proteomics [13] or metabolomics [14]. Currently, only assays analyzing CTCs and ctDNA have been approved by the US Food and Drug Administration (FDA). CTC enumeration (CellSearch®, Menarini Silicon Biosystems, Huntingdon Valley, Pennsylvania USA) is FDA-approved and has a prognostic value in metastatic breast [15,16], colorectal [17], and prostate cancers [18], but its clinical utility is still to be demonstrated. The FDA also approved the cobas® EGFR Mutation Test v2 (Roche Molecular Diagnostics, Pleasanton, California, USA) to detect *EGFR* mutation in the cell-free DNA (cfDNA) of lung cancer

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patients [19,20]; and Epi proColon® (Epigenomics, Berlin, Germany) to assess the methylation status of the *SEPT9* promoter in cfDNA to screen patients for colorectal cancer [21–23].

The gold standard for cancer diagnosis remains tissue biopsy as, in contrast to liquid biopsy, it provides histological features of the tumor in addition to molecular characterization. However, obtaining a tissue biopsy can sometimes be challenging and technically difficult due to disease localization. LB is easily accessible, less invasive and more comfortable for the patient. At the molecular level, LB should theoretically be able to capture intra-patient tumor molecular heterogeneity as it allows ctDNA released from multiple tumor regions to be analyzed. Therefore, it should be able to reflect the different genomic alterations between the primary tumor and metastatic lesions [24]. In addition, LB may also address tumor heterogeneity over time [25] by enabling multiple samples to be taken over the course of the disease.

With ease of sampling making it clinically attractive, the field of LB is rapidly evolving. Awareness of its potential applications, technical limitations and supportive evidence is now useful knowledge for clinicians. In this review, we provide an overview of the potential use and technical challenges of ctDNA and then focus on the available data of this new diagnostic tool in squamous cell carcinoma of the head and neck.

Potential clinical applications of ctDNA

ctDNA is cell-free DNA (cfDNA) from tumoral origin that is essentially derived from apoptotic or necrotic tumour cells [26,27], although active release is also implicated [28]. The analysis of ctDNA can provide information on tumor-specific genomic alterations including somatic mutations, copy number alterations, fusions or epigenetic alterations. Below, we briefly describe the potential clinical applications of ctDNA.

Cancer diagnosis and early detection

The first potential application of ctDNA is cancer screening and early diagnosis in the hope that earlier treatment will lead to improved outcome. Some studies have focused on quantitative analyses of cfDNA as an indicator of tumor burden. Indeed, the level of cfDNA has been shown to be higher in cancer patients compared to healthy controls [29,30]. Moreover, cfDNA levels seem to correlate with tumor stage. However, knowing only the quantity of cfDNA is not particularly informative as other parameters, including inflammation, exercise, autoimmunity, smoking, pregnancy and heart dysfunction, can influence cfDNA levels [31]. In contrast, ctDNA is more attractive as it detects genomic alterations that are tumor-specific, and this has been explored in various studies. Bettegowda et al. reported ctDNA detection across various cancer types in 82% of patients with stage IV and in 47% of patients with early stage disease [32]. Using the targeted error correction sequencing (TEC-Seq) approach, Phallen et al. detected somatic mutations in the plasma of 71%, 59%, 59%, and 68% of patients with early-stage (I/II stage) colorectal, breast, lung or ovarian cancers, respectively [6]. Another blood-based test, CancerSEEK, which combines mutations in cfDNA and circulating proteins, was positive in 70% of 1005 patients with non-metastatic clinically detected cancers (43%, 73%, and 78% in stage I, II and III, respectively).

Changes in methylation patterns are early events in carcinogenesis and may also be useful in cancer detection. Aberrant circulating DNA methylation patterns have been shown to be a potential tool for the early diagnosis of disseminated breast cancer [33] or ovarian cancer [34]. Copy number alterations detected by whole genome sequencing have led to the detection of early ovarian cancer [35]. These studies outline the potential application of early cancer detection through ctDNA analysis. However, important challenges remain before this evolving diagnostic field can be confidentially implemented. Clonal hematopoiesis, or the presence of somatic mutations within nonmalignant hematopoietic cells, is described in up to 10% of individuals aged over 65 years and this could lead to false positive results [36,37]. Furthermore, some studies have described the presence of somatic mutations in normal cells from apparently healthy individuals [38,39]. Recently, results of a substudy from the Circulating cell-free Genome Atlas (CCGA) study on 2301 participants (1422 cancer, 879 non-cancer), showed that targeted methylation technology had a sensitivity ranging from 59 to 86% (across cancer types and stages) with a 99% specificity [40]. This low false-positive rate is critical before population-based screening test can be implemented. Using a novel multivariate cancer risk score (NCRS) model that combines shallow whole genome sequencing of cfDNA and protein markers, Mao et al. reported 71.8% sensitivity and 97.7% specificity for detection of multiple cancer types [41].

Prognosis and detection of minimal residual disease

ctDNA can be used to detect minimal residual disease (MRD), defined as the persistence of microscopic cancer after treatment that cannot be detected with current medical imaging modalities [42]. MRD is correlated with an increased risk of disease recurrence and poorer outcome. Several studies have shown the feasibility of ctDNA to detect MRD after curative treatment in various cancer types including breast [43] and colon cancers [44]. MRD detection often requires the identification of a somatic mutation in the solid tumor biopsy at baseline that will be followed longitudinally by digital polymerase chain reaction (PCR) [43] or next generation sequencing (NGS)-based assay [44]. However, Chaudhuri et al. have shown that ctDNA may be useful to detect MRD even without prior knowledge of a tumor's mutational status [7]. In a post-treatment sample, ctDNA detection was reported in 94% of lung cancer patients with disease recurrence. In these studies, ctDNA detection often preceded clinical recurrence.

Characterization of molecular landscape, precision medicine and treatment selection

Several studies have shown that ctDNA has the potential to reflect the mutational landscape of a tumor. High concordance rates between liquid and solid biopsies have been reported for driver oncogenes, including *KRAS* and *EGFR* [45,46]. This can be of particular interest to identify actionable mutation(s). For example, many efforts have been made to detect *EGFR* mutations in the plasma of patients with lung cancer as obtaining biopsies can be challenging in lung cancer and *EGFR* mutations are predictive of response to EGFR tyrosine kinase inhibitors (TKI). Although the sensitivity of *EGFR* mutation detection in plasma was reported to be only 65.7% [45], these tests can avoid invasive tumor biopsies to a significant number of patients.

Monitoring of treatment and emergence of resistant clones

The short half-life of ctDNA and the ease of sampling make LB an interesting tool for monitoring treatment response longitudinally. ctDNA dynamics have been shown to correlate with tumor response in many cancers [8,47,48]. In breast cancer, ctDNA outperformed cancer antigen 15-3 in predicting response to chemotherapy and relapse [8]. A study in non-small cell lung cancer found that ctDNA could distinguish between residual disease and treatment-related imaging modifications [48]. This illustrates the complementary role that ctDNA can provide to imaging for disease monitoring. Somatic mutation analysis can also provide information regarding potential resistance mechanism(s) such as the emergence of *RAS* mutations in colorectal cancer patients [49] treated with EGFR-targeted therapies, or the appearance of the *EGFR* T790M mutation in lung cancer patients that confers resistance to gefitinib and erlotinib [50].

Table 1
Overview of sequencing technologies for cfDNA analysis (non-exhaustive).

Extent of analysis	Examples of technology	Assay limit of detection	Potential clinical applications
Single mutation or limited multiplex panel	BEAMING [88]	0.01%	Detection of hot-spot mutations [50]
	ddPCR [52,53]	0.001%	Disease monitoring and detection of minimal residual disease [43]
Targeted panel gene (10 kb to 50 Mb)	Standard NGS [55,89]	1%	Mutational landscape [91]
	Safe-SeqS [57]	0.1%	Detection of actionable mutations [55,81]
	SiMSen-Seq [90]	0.1%	Disease monitoring and detection of minimal residual disease [7,44,92]
	CAPP-seq [48]	0.02%	
	iDES	< 0.01%	Detection of *de novo* mutations implicated in resistance mechanisms
Whole Exome	Hybrid-capture exome sequencing [60]	> 5%	Detection of resistance mechanism [60]
Whole Genome	Plasma-seq [61]	5–10%	Genomic landscape [61]

BEAMING, bead-emusion-amplification-magnetics; CAPP-seq, personalized profiling by deep sequencing; ddPCR, digital droplet PCR; iDES, integrated digital error suppression kb, kilobases Mb, megabases; NGS, next generation sequencing; PCR, polymerase chain reaction; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing.

Technical aspects of ctDNA somatic mutation analysis

The optimal sequencing technique should consider the end goal(s) of ctDNA analysis. The main challenge with ctDNA analysis is that the proportion of ctDNA can sometimes be extremely low (< 1%) [47]. Therefore, the detection of MRD, for example, will require a highly-sensitive technique that is able to detect ctDNA at very low proportions.

Table 1 provides an overview of ctDNA sequencing approaches. There are multiple techniques to analyse ctDNA and these vary in the number of genomic alterations covered by the sequencing technique and in the limit of detection. The limit of detection is the lowest mutant allele fraction or the lowest allele concentration that will confidently discriminate the relevant mutations from the background noise [51]. Below this threshold, the analysis will be hampered by technical artefacts such as PCR or sequencing errors. The mutant allele fraction (expressed in percentage) is the ratio of the number of mutant reads divided by the total number of reads at a specific genomic position number, and the mutant allele concentration (expressed in copies per mL) is the number of mutant DNA fragments per unit volume.

The extent of ctDNA analysis can vary from a single mutation or a limited multiplex panel to whole genome.

The cobas® *EGFR* mutation test v2 is an example of an allele-specific real-time PCR-based test which focuses on *EGFR* mutation detection with a limit of detection of 25 copies/mL. In digital-PCR based technologies, like digital droplet PCR (ddPCR) [52,53] or bead-emulsion-amplification-magnetics (BEAMing) [54], the partitioning of the DNA sample into thousands of individual fractions before PCR-amplification enables absolute quantification without standard curve and a lower limit of detection compared to real-time PCR. These techniques, however, only have the potential to detect a limited number of mutations of interest. They can be used for hotspot mutation detection [50] or detection of MRD [43]. In the latter, knowledge of the presence of specific mutations in the tumor tissue biopsy of each individual patient is required. Advantages of these techniques include the absence of bio-informatic analysis and a rapid turn-around time.

Targeted NGS techniques, using PCR amplicons or hybrid-capture, enable broader gene panels to be targeted. For standard NGS techniques, detection is limited to variants with a mutant allele fraction of 1% because of the background error rate [55–57]. These errors can be reduced by implementing barcode based approaches or specific bioinformatic tools. For instance, the Safe-sequencing system (Safe-SeqS) [57] uses unique molecular identifiers (UMI) or molecular barcodes that will tag sequences from the same DNA fragment to identify possible errors. Personalized profiling by deep sequencing (CAPP-seq) [48] is a capture-based enrichment strategy that includes a specialized bioinformatic workflow. It can be further improved by the coupling of integrated digital error suppression (iDES) to eliminate artefacts observed in cfDNA sequencing [58]. These techniques do not need patient-specific tumor analysis and have the advantage over standard NGS approaches [55,59] to offer a lower limit of detection.

Finally, whole exome or whole genome sequencing offers the most comprehensive approach but at the cost of a lower limit of detection estimated at 5% because of the lower sequencing depth [60]. Shallow whole genome sequencing with a 0.1–0.2 × low sequencing coverage is used for copy number alteration detections [61,62]. The drawback of NGS-based techniques is the bio-informatic expertise needed to analyze the data and the higher cost compared to digital PCR-based techniques.

cfDNA in SCCHN

Some studies have explored LB as a diagnostic, prognostic or monitoring tool for SCCHN, but data are limited compared to other cancers and there is currently no clinical application. LB could, however, be of interest at different stages of SCCHN disease (Fig. 1). For instance, 40–50% of patients treated with curative intent will recur. The suspicion of local relapse is currently based on physical examination, including flexible head and neck fiberoptic endoscopy and imaging. The differential diagnosis with treatment-induced fibrosis or radionecrosis can be difficult, leading to delayed diagnosis and treatment. Additionally, in some cases of locoregional recurrence without distant metastases, salvage surgery or (chemo)radiation can still be curative. Therefore, early diagnosis of locoregional recurrence is primordial. ctDNA could be useful to distinguish the difference between local relapse and treatment-related complications, and to detect MRD, with the ultimate goal of identifying patients who need additional treatment.

Another interesting field for LB is precision medicine. The characterization of the SCCHN molecular landscape has identified potential therapeutic targets with new treatment opportunities [63,64]. SCCHN is a heterogenous disease, and multiple molecular assessments during the disease course may be required to adapt the treatment strategy. Identifying potentially actionable alterations through LB could be an attractive alternative when obtaining a biopsy is challenging or not feasible.

Blood-based DNA analysis for SCCHN includes not only the detection of tumor-specific mutations but also the detection of the human papillomavirus (HPV) DNA for HPV-positive oropharyngeal cancer [65–67]. Furthermore, different studies have investigated ctDNA microsatellite alterations [68,69], methylation levels [70,71], and copy number aberrations [72].

ctDNA detection trough somatic mutation analysis

Somatic mutations in ctDNA can be analyzed using two different approaches: a *targeted approach* and a *tumor-agnostic approach*. The

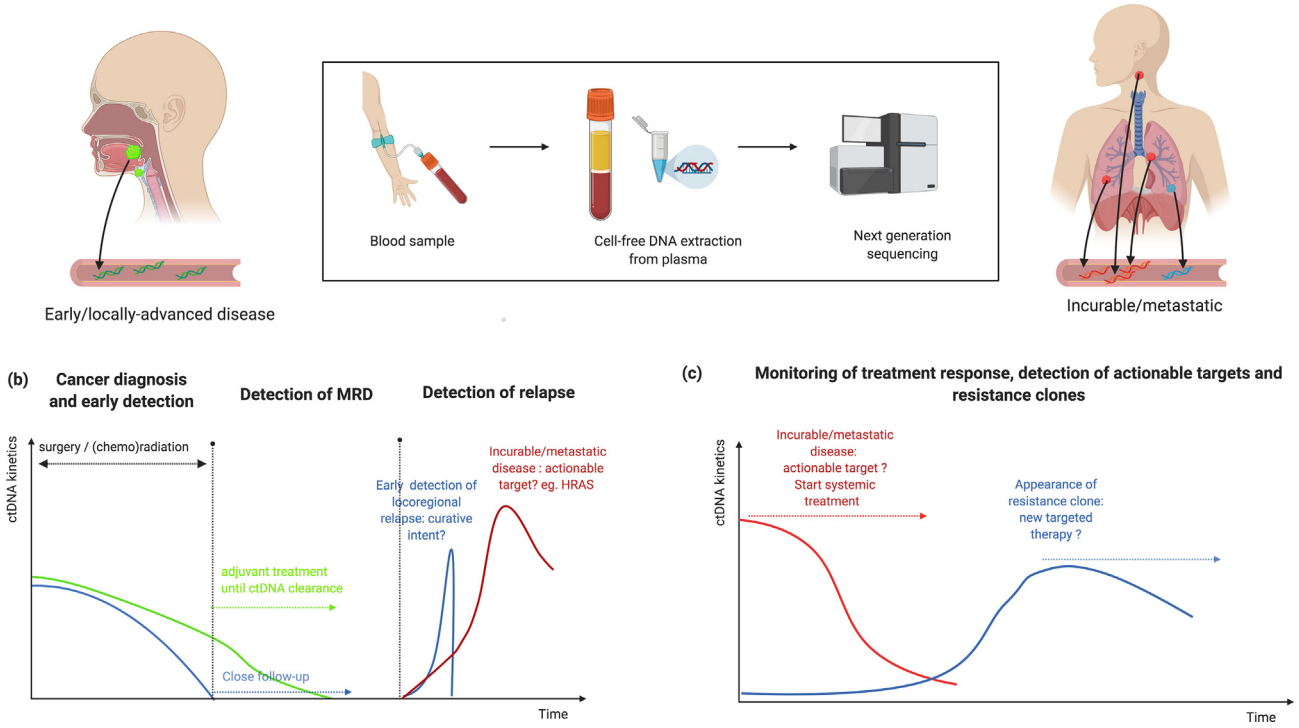

Fig. 1. Potential clinical applications of circulating tumor DNA at different stages of SCCHN disease The figure shows how cancer lesions shed tumor DNA into the blood circulation and how ctDNA could reflect tumor heterogeneity in case of multiple tumor clones. The box in the middle illustrates the flow diagram for ctDNA analysis. (a) The graph shows the potential clinical applications of ctDNA kinetics during the curative setting. The blue and the green lines represent ctDNA kinetics of 2 different patients undergoing curative treatment for locally-advanced SCCHN. Both patients have ctDNA detected at baseline. After treatment, the blue curve show ctDNA clearance, reflecting possibly that this patient is cured and could benefit from a close follow-up without adjuvant treatment. The green curve however, could potentially indicate minimal residual disease for this patient and guide an adjuvant treatment. Serial longitudinally ctDNA could lead to earlier detection of loco-regional relapse that is still curable. In case of incurable/metastatic relapse, ctDNA could identify potentially actionable alterations. (b) The graph shows how ctDNA kinetics could guide treatment for incurable/metastatic SCCHN; monitoring treatment response (red curve) to systemic treatment, identify potential targets as well as the emergence of resistance clones (blue curve).

targeted approach identifies genomic alteration(s) in tumor tissue that can be used as marker(s) for ctDNA presence through different high-sensitive sequencing techniques. Another approach is the *tumor-agnostic approach* or *non-targeted approach*, whereby ctDNA is analyzed without prior knowledge of the mutational status of the tumor [7]. This could also be of high interest when tumor biopsies are not feasible or difficult.

Targeted approach

Different studies in SCCHN have used a targeted approach to detect ctDNA in plasma based on somatic mutation(s) previously identified in the tumor (Table 2). The main clinical applications for this approach are baseline ctDNA detection and disease monitoring or minimal residual disease detection.

The largest study was reported by Wang et al. [73]. The group explored the detection of tumor-specific DNA in the saliva and/or plasma of 93 SCCHN patients including 20 patients (22%) with early stage (I or II) disease. At least one genomic alteration was detected in the tumor of each patient including the detection of HPV16 DNA in 30 patients (32%), a somatic mutation in genes commonly altered in SCCHN in 58 patients (62%), or another driver mutation/translocation in the last five patients. Subsequently, digital PCR or Safe-SeqS for the detection of HPV DNA and point mutations/translocation, respectively, was applied to the saliva (n = 93) and plasma (n = 47). The investigators detected ctDNA in 87% of the 47 patients whose plasma was tested. This detection rate increased to 96% when both plasma and saliva were combined. Importantly, it was demonstrated that a limited panel of genes, containing HPV DNA 16 sequences and the most common altered genes in SCCHN (*TP53*, *PIK3CA, CDKN2A, NOTCH1*), led to the detection of ctDNA in 95% of SCCHN. Therefore, ctDNA detection seems feasible in SCCHN when highly sensitive techniques are used.

In the same patient cohort, 14 patients with sequenced plasma had locoregional recurrent disease. ctDNA was detected in all of them, highlighting the potential of ctDNA to help diagnose recurrent disease. Although it was not the main purpose of their work, Wang and colleagues also examined the follow-up samples of nine patients who had detectable ctDNA before surgery. In three of these patients, tumor DNA was detected in the saliva or plasma after surgery and before clinical relapse, but none of the five patients without recurrence had detectable tumor DNA. The same investigators also showed on this limited number of patients that those with undetectable tumor DNA in their saliva/plasma after surgery had a better disease-free survival, suggesting a prognostic value.

In contrast, Perdomo [74] et al. detected ctDNA in only 42% of patients using a targeted approach. As cfDNA samples used for this analysis were up to 10 years old, sample storage and handling may explain these modest results.

Van Ginkel et al. [75] focused on the *TP53* gene. His group performed *TP53* testing in surgically resected primary tumor samples of six SCCHN patients, and designed mutation-specific ddPCR assays to detect the mutation in plasma. The mutation was detected in all six plasma samples with an allele frequency down to 0.01%, demonstrating that a highly sensitive PCR-based technique could be used as a post-treatment monitoring tool in SCCHN.

The first study focusing on detection of minimal residual disease was recently published [76]. Seven patients, with identifiable mutations in the primary tumor, were tested for the presence of these same patient-specific mutations in their plasma. Plasma-sequencing assays (SiMSen-Seq) were designed based on the mutations identified in the tumors (2–7 mutations per patient). In six patients (86%), ctDNA mutations were detected at baseline and these were followed for up to

Table 2
Selected studies focusing on ctDNA in SCCHN using a targeted approach.

Study	N	Sample	Analysis	Technique(s)	Outcome
Wang et al. [73]	93 SCCHN Stage I-II (n = 20) Stage III-IV (n = 73) Oral cavity (n = 46) Oropharynx (n = 34) Larynx (n = 10) Hypopharynx (n = 3)	Tumor, plasma and/or saliva	Somatic mutations or HPV gene	Identification of at least 1 genetic alteration in each tumor (HPV16 DNA detection (n = 30), somatic mutation of one of the following genes *TP53, PIK3CA, CDKN2A, FBXW7, HRAS, and NRAS*) (n = 58) or other driver mutation/translocation (n = 5)) DNA detection in saliva/plasma: –Digital PCR for HPV16 DNA (*E7* gene) – Safe-SeqS for translocations and point mutations	ctDNA detection: –plasma alone: 87% (n = 47) –saliva alone: 76% (n = 93) (100% for oral cavity) –plasma and saliva: 96% (n = 47) Detection of tumor DNA in saliva or plasma post-surgically in 3 patients before clinical recurrence, but in none of the 5 patients without recurrence.
Van Ginkel et al. [75]	6 SCCHN (stage II-IV)	Tumor Plasma	Patient-specific *TP53* mutation detected in each tumor	*TP53* determined in surgically resected primary tumor samples Mutation-specific ddPCR assays for pre-treatment ctDNA detection	T*P53* mutations detected in all plasma samples with fractional abundance down to 0.01%
Egyud et al. [76]	8 SCCHN at diagnosis	Tumor Plasma	Somatic mutations	Tumor sequencing to identify specific mutations ctDNA analysis: SiMSen-Seq (range 2–8 mutations per patient)	Baseline ctDNA positive in 6 patients. ctDNA detection in 3/4 patients with recurrence.
Perdomo et al. [74]	36 HPV-negative SCCHN carrying known mutation in the tumor	Tumor and Plasma	Targeted approach to detect mutations in plasma, previously detected in the tumor	Plasma: Targeted sequencing (amplicon-based, ion torrent™ proton sequencer) in 5 genes (*TP53, NOTCH1, CDKN2A, CASP8, PTEN*)	ctDNA detection in 15/36 (42% of patients) Across 36 SCCHN patients, 65 mutations in 5 genes were detected in the tissue samples, of which only 28% were detected in the plasma.

AF, allele frequency; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; HPV, human papillomavirus; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing; PCR, polymerase chain reaction; SCCHN, squamous cell carcinoma of the head and neck.

Table 3
Selected studies focusing on ctDNA in SCCHN using a non-targeted approach.

Study	N	Sample	Analysis	Technique(s)	Outcome
Mazurek et al. [30]	200 SCCHN	Plasma	cfDNA quantification HPV16/18 DNA detection Mutations in *EGFR/KRAS*	DNA concentration in plasma by Taqman- based TERT amplification Taqman-based E6/E7 HPV16 and HPV18 DNA TaqMan-MGB assay for *KRAS* G12C and *p.E746—A750del* mutation of *EGFR*	Higher cfDNA levels in OPSCC, in clinical N2-N3, and in stage IV 14% of HPV-positive based on plasma analysis No detection of the pre-defined *EGFR/KRAS* mutations
Braig et al. [79]	20 SCCHN treated with cetuximab, 13 with PD under cetuximab	Serum (post-cetuximab) Tumor (baseline, before cetuximab)	*RAS* mutation and epitope-modifying *EGFR* mutations	NGS to screen *EGFR* exon 12, *KRAS/NRAS* exon 2,3,4 and *HRAS* exon 2/3 (mean number of > 20,000 reads per exon)	6/13 pts with progressive disease during cetuximab-based therapy acquired activating *RAS* mutations detectable in cfDNA No *EGFR* ectodomain mutations were found
Perdomo et al. [74]	37 stage III-IV SCCHN	Tumor, plasma, and oral rinses	Independent *TP53* mutation detection in each sample type: tumor, plasma and oral rinses	Sequencing of entire coding region of *TP53* gene (Proton sequencer, 400X tumor, 3000x on plasma)	*TP53* mutations in 28/37 of solid tumor cases (76%), but only in 3 cfDNA samples (8.1%) *TP53* mutations in oral rinses was higher for oral cavity and oropharynx Low concordance between tumor, oral rinses, and plasma
Porter et al. [93]	60 HNC OPSCC (n = 21) Salivary gland and thyroid cancer	Blood (not otherwise specified) CtDNA data compared to tumor NGS data when available (n = 29)	70 gene panel	Guardant360, a 70-gene ctDNA NGS platform	Most frequent mutations: *TP53* (98%), *PIK3CA* (43%), *NOTCH1* (38%), and *ARID1A* (36%). 73% (n = 22) of patients had alterations identified in ctDNA that were not present in the tumors Actionable mutations were identified in 66% of SCCHN and in 50% salivary gland cancer patients
Soulières et al. (2018) [95]	112 recurrent/metastatic SCCHN, after platinum	Plasma archival tumor	542 gene panel 44 gene panel	Targeted NGS with a panel of 542 genes	Molecular landscape of ctDNA: most frequently mutated genes: *TP53, FAT1, TET2, KMT2D, PIK3CA, NOTCH1, NFE2L2, NOCTH2, CCND1, CDKN2A* Concordance between archival tissue and ctDNA: 89% for HPV, 63% for *TP53* status
Schmidt et al. [80]	29 treatment naïve SCCHN patients (stage III-IV)	Plasma	*PIK3CA* p.E545K mutation	PlexPrime™ and PlexZyme™ technology in real-time PCR, allele-specific amplification technology	*PIK3CA* E545K detection in 9/29 patients (31%)
Ping Wu et al. [94]	27 SCCHN	Plasma and saliva pre- and post-surgery Tumor	1021 gene panel	Targeted NGS with a panel of 1021 genes	Tumor-specific mutations observed in 70% in plasma and 52% in saliva ctDNA more likely detected in saliva from oral cancer (83%) versus 46% and 40% in pharynx and hypopharynx DFS lower in patients with detectable postoperative cfDNA compared to undetectable
Fostira et al. [81]	Cohort 1: 54 LA-SCCHN Cohort 2: 15 metastatic SCCHN	Cohort 1: Plasma (baseline, end of treatment and PD) Tissue (baseline) Cohort 2: Plasma (baseline and post 2nd cycle)	*TP53, CDKN2A, HRAS* and *PIK3CA*	Plasma and tissue: Sequencing with SafeSEQ (*TP53, CDKN2A, HRAS* and *PIK3CA*)	Cohort 1: ctDNA detection in 56% of patients whose tumor harbored mutation at baseline. In 3 patients, detection of *HRAS* mutations in plasma, not detected in tissue at baseline At progression, new mutations were detected in 40%. Cohort 2: ctDNA detection in 93% of metastatic patients prior to and during treatment with nivolumab. 3 patients had emergence of *HRAS* mutations during treatment
Galot et al. [82]	39 SCCHN: locoregional recurrent (n = 19) metastatic (n = 20)	Plasma at baseline Matched tumor tissue (n = 18)	Custom gene panel (604 genes)	Plasma and tumor: NGS (Illumina HiSeq4000, 1000X))	ctDNA detected in 51% of patients (70% of metatstatic patients versus 30% of locoregional recurrent, p = 0.025) concordance: 26% of the plasma variants (14/54) were not detected in the matched tumors 81% of the variants (168/208) identified in the solid tumors were not detected in the plasma. Detection of 3 different potentially actionable *PIK3CA*

(*continued on next page*)

Table 3 (*continued*)

Study	N	Sample	Analysis	Technique(s)	Outcome
					mutations in the plasma of patients with no tumor available

cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; HPV, human papillomavirus; DFS, disease-free survival; HNC, head and neck cancer; LA, locally-advanced; PCR, polymerase chain reaction; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PD, progressive disease; PCR-based barcoding of DNA for sensitive mutation detection using sequencing; SCCHN, squamous cell carcinoma of the head and neck; TERT, human telomerase reverse transcriptase.

18 months after therapy. Out of the four patients diagnosed with clinical recurrence, three had at least one mutation detected in their longitudinal plasma samples.

All these studies demonstrate that targeted approaches based on high-sensitive techniques are feasible with promising potential clinical applications.

Tumor-agnostic approach

Non-targeted or tumor-agnostic approaches sequence cfDNA without knowing the genomic alterations of the corresponding solid tumor. The main clinical applications are characterization of the molecular landscape, identification of actionable events and investigation of treatment resistance mechanisms.

Table 3 provides an overview of tumor-agnostic studies performed in SCCHN. Mazurek et al. [30] studied the cfDNA of 200 patients with the primary aim of correlating cfDNA quantification with clinical characteristics. Higher cfDNA levels were found in oropharyngeal cancer as well as in clinical N2-N3 and in stage IV disease. The investigators also looked for plasmatic HPV16/18 DNA, *KRAS* G12C and *EGFR* mutations. HPV DNA was detected in 14% of patients but no *KRAS* or *EGFR* mutations were detected; these two latter alterations are, however, not frequent in SCCHN. Perdomo et al. [74] also explored the non-targeted approach by sequencing the *TP53* gene independently in the tumor, saliva and plasma of 37 patients with late-stage SCCHN. As expected, *TP53* mutations were detected in 28/37 of solid tumor biopsies (76%), but surprisingly, these mutations were only observed in three plasma samples. Only in one patient was the same mutation detected in tumor, plasma and oral rinses. Again, the low plasmatic detection rate observed by Mazurek and colleagues might be explained by the long storage of plasma samples. An important finding of the study was the identification of *TP53* mutations in the oral rinses of a few non-cancer individuals with an allele frequency ranging from 0.001 to 0.004%. The detection of *TP53* mutations has been previously reported in the plasma, peritoneal fluid and skin of healthy controls [38,39,77,78]. Clonal hematopoiesis has also been reported as potential origin of false-positives [36]. These findings need to be taken into account when considering ctDNA for early cancer detection.

A targeted NGS-approach has been used to screen for the emergence of specific *EGFR* ectodomain or activating *RAS* mutations in patients treated with cetuximab-based therapy [79]. The primary tumor at baseline before cetuximab treatment was analyzed for the same specific mutations. The appearance of an activating *RAS* mutation was found in the cfDNA of 6/13 (46%) patients that had progressive disease during cetuximab-based treatment, while all the tumor baseline samples of these patients were *RAS* wild type. This underlines the possibility to detect hotspot mutations using ultra-deep NGS testing as well as *de novo* mutations possibly implicated in treatment resistance. The detection of a hotspot *PIK3CA* mutation was also reported in 9/29 (31%) patients with treatment-naïve SCCHN using an allele specific amplification technique [80]. This could be of clinical interest as *PIK3CA* p.E545K is a potentially targetable event, and the technique that is used has a short turn-around time. Recently, another group detected *HRAS* mutations in three patients with locally-advanced SCCHN that were not present in the baseline tumor samples [81]. Furthermore, the investigators described the appearance of *HRAS* mutations in three other patients who progressed on nivolumab. Such data could potentially inform a targeted treatment strategy as SCCHN patients harboring a *HRAS* mutation could potentially benefit from tipifarnib, a farnesyltransferase inhibitor [63].

We have explored the potential of liquid biopsy for tumor mutational profiling of SCCHN using targeted NGS (Ilumina HiSeq4000, custom panel of 604 genes with a median coverage of 1000 X) [82]. Although liquid biopsy did not reflect the complete mutation profile of the tumor, we identified some potential targetable events for patients with no tumor biopsy available. The concordance between the solid and liquid biopsy was low due to the limited sensitivity of the standard NGS approach.

Table 4
Selected studies focusing on cfDNA methylation patterns, microsatellite alterations or copy number aberrations.

Study	N	Sample	Analysis	Technique(s)	Outcome
Mydlarz et al. [71]	100 SCCHN 50 healthy controls	Serum	*EDNRB*, *CDKN2A* and *DCC* DNA methylation	Quantitative methylation specific PCR (qMSP)	*EDNRB* hypermethylation in 10% of SCCHN patients versus none in the control group
Schrock et al. [70]	649 Training study: 284 SCCHN/122 controls Validation study: 141 SCCHN/102 controls	Plasma	*SHOX2* and *SEPT9* DNA methylation	Methylation was determined using SHOX2/SEPT9/ACTB (actin beta) triplex quantitative PCR	Training study: Sensitivity: 59% Specificity: 96% Higher risk of death in patients with positive SEPT9 and SHOX2 plasma levels compared to negative patients Disease relapse detected in 47% of patients before clinical recurrence Validation study: Sensitivity: 52%, Specificity: 95%, OS prediction: patients with positive SEPT9 and SHOX2 plasma levels were at higher risk of death compared to methylation-negative patients Correlation with tumor and nodal category
Hamana et al. [69]	64 OSCC	Serum: preoperatively, postoperatively, and 4 weeks post-operatively Tumor samples and normal controls	Microsatellite blood assay (9 microsatellite markers)	PCR	At least 1 allelic imbalance in cfDNA in 87% (33 of 38) of the patients who had an allelic imbalance detected in tumor DNA
Nawroz-Danish et al. [68]	152 SCCHN	Tumor Serum	Microsatellite blood assay	PCR	45% (68/152) had microsatellite alterations of ctDNA identical to the corresponding tumor DNA. Patients with microsatellite alterations detectable in their cfDNA had higher rate of distant metastasis (16.2% (11/68) versus 6% (5/84), RR = 2.7) OS did not differ between positive and negative serum tests
Schirmer et al. [72]	103 SCCHN without distant metastasis Control group: 142 tumor-free individuals	Plasma	ctDNA-derived CNA	Low-coverage NGS	CNAs were combined in a genome-wide copy number instability score (CNI): ctDNA analysis to detect tumors: –specificity :95% –sensitivity: 46% (pT1), 94% (pT4) CNI above the median (> 72), OS was worse

CDKN2A, cyclin-dependent kinase inhibitor 2A ;cfDNA, cell-free DNA; CNA, copy number alterations; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; DCC, deleted in colorectal carcinoma; *EDNRB*, endothelin receptor type B; HPV, human papillomavirus; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing; SHOX2, *Short stature homeobox 2*; SEPT9, *Septin 9* ; SCCHN, squamous cell carcinoma of the head and neck; PCR, polymerase chain reaction; qMSP, quantitative methylation specific PCR; OS, overall survival; RR, relative risk.

Analysis of cfDNA for methylation patterns, microsatellite alterations or copy number alterations

Other cfDNA analyses can be performed, including the investigation of methylation patterns (Table 4). Mydlarz et al. [71] investigated the ability of three methylation probes (endothelin receptor type B (*EDNRB*), deleted in colorectal carcinoma (*DCC*), and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) hypermethylation probes) to detect altered methylation in serum samples from 100 patients with untreated SCCHN and 50 healthy controls. *EDNRB* hypermethylation was reported in the serum of 10% of patients whilst none was observed in the control samples, highlighting a high specificity for this test but limited sensitivity as a biomarker for SCCHN. In contrast, Schrock et al. [70] reported that *Short stature homeobox 2* (*SHOX2*) and *Septin 9* (*SEPT9*) DNA methylation had a higher sensitivity (52%) and a specificity of 95%. They also found that methylation levels predicted overall survival (OS) and correlated with tumor and nodal category.

Different studies have investigated microsatellite alterations in ctDNA [68,69]. Nawroz et al. detected microsatellite alterations in the serum of 47% patients, and these were identical to the ones seen in the corresponding tumors. Patients with microsatellite alterations detectable in their cfDNA had a higher rate of distant metastasis but there was no correlation with OS. Hamana et al. [69] also looked at the microsatellite status of patients with SCCHN and analyzed pre- and postoperative samples. Allelic imbalances (AI) were observed in 38 out of 64 tumor tissues. They detected at least one AI in the serum of 33 of these 38 patients. All the serum AI patterns were matched with the corresponding tumor. Serum AI were detected preoperatively in 44% (28 of 64) and postoperatively in 20% (13 of 64) of patients. In the preoperative positive patients, 20 (71%) had no detectable ctDNA postoperatively and none recurred. Two patients with detectable post-operative ctDNA died within 44 weeks of surgery.

Finally, copy number alterations (CNA) can also be analyzed through cfDNA. Schirmer et al. analyzed ctDNA derived CNA in SCCHN patients with low-coverage NGS [72]. They calculated a copy number instability score (CNI) from the number of copy imbalances as a general measure of the genomic instability that occurs in the tumor and in the concentration of ctDNA. They found sensitivity for tumor detection that ranged from 46% for pT1 tumors to 94% for pT4. Patients with a CNI above the median (> 72) had worse survival with an OS survival rate at three years of 62% compared to 90% in patients with CNI < 72.

Circulating HPV DNA

In HPV-related OPC, there is expression of viral-specific oncogenes (E6 and E7) [83]. Some studies have focused on the detection of these viral oncogenes in LB to use HPV cfDNA as a biomarker for HPV-positive OPC (Table 5). Ahn et al. reported a sensitivity and a specificity of 76% and 100%, respectively, when HPV DNA analysis of pretreatment saliva and plasma samples were combined [65]. The sensitivity of LB alone was 67.5%. More recently, Chera and colleagues reported a sensitivity of 89% with a more sensitive digital PCR technique [84].

HPV cfDNA levels seem to correlate with tumor burden as patients with stage IV and high N category had a higher rate of detectable pretreatment serum HPV DNA as reported by Dahlstrom et al.[65] This was confirmed by Hanna et al. [85] who tested HPV cfDNA in 17 patients with R/M disease. The median HPV cfDNA levels increased with disease spread: 4.6 copies/mL in locoregional disease, 163 copies/mL in pulmonary-only disease, and 452.8 copies/mL in extra-pulmonary disease [85]. In this study, the median plasma HPV cfDNA level negatively correlated with survival, suggesting a prognostic value. The opposite was however shown in another study where low pre-treatment HPV cfDNA was associated with worse outcome, possibly related to lower tumor HPV copy numbers and greater likelihood of HPV integration which are adverse tumor genomic features [84].

Finally, evidence is emerging that cfDNA could be used for monitoring. One study reported that HPV DNA detected in post-treatment saliva was associated with a higher risk of recurrence [65]; in two other studies, changes in HPV cfDNA levels confirmed treatment response or progression [85,86]. Recently, Chera and colleagues showed in a prospective biomarker trial that a rapid clearance profile of HPV cfDNA, defined as having a high baseline copy number (> 200/mL) and $> 95\%$ clearance by day 28 of CRT, could predict disease control in patients with HPV-positive OPSCC treated with definitive CRT [84]. However, patients with detectable HPV cfDNA at the end of week 6 of CRT did not have a higher incidence of residual disease or recurrent disease.

Saliva

Most of the studies that investigate LB in SCCHN are based on blood analyses, although another interesting “liquid” in this disease, as previously mentioned, is saliva. Saliva can contain molecular biomarkers through ultrafiltration from blood, passive diffusion, or active transport [87]. Moreover, DNA can be released immediately into the upper aerodigestive tract by the necrosis of SCCHN epithelial cells. In the study by Wang et al. tumor DNA was detected in 76% of saliva samples ($n = 93$). Combined analysis of saliva and plasma increased the detection of ctDNA from 87% to 96% compared to plasma analysis alone. This same trend was observed for the detection of HPV cfDNA when plasma and saliva samples were combined [65]. The sensitivity of ctDNA detection in saliva seems to be site-dependent. Tumor DNA detection was achieved in 100% of oral cavity cancers but only in 47%, 70% and 67% of oropharynx, larynx, and hypopharynx cancers, respectively [73]. Perdomo et al. [74] described a *TP53* mutation detection rate in oral rinses that was also higher in oral cavity (54.2%) and oropharynx (60%) cancers compared to larynx cancer (16.7%).

Conclusion

Although the literature on cfDNA analysis for SCCHN is scarce compared to other tumors, preliminary results seem to hold promise for the future. Early evidence suggests that highly sensitive techniques on limited gene panels could be useful to detect ctDNA in a substantial proportion of patients with SCCHN. This is of particular interest to detect minimal residual disease, for which proof of concept has already been shown. The next step would be to evaluate the clinical utility of ctDNA detection in this setting to select upfront patients who would benefit from complementary or adjuvant therapy after primary treatment.

Moreover, exciting results have emerged regarding the detection of potentially targetable events through liquid biopsy, like *PIK3CA* or *HRAS* mutations, for which tipifarnib may be a potential therapeutic option [63]. Some of these mutations were not present in the baseline tumor sample and others emerged during therapy [79,81]. Liquid biopsy could thus be an attractive tool in the future, complementing tissue biopsy to capture tumor heterogeneity, or replacing it when a biopsy is not feasible or challenging.

To conclude, large-scale prospective studies are required to further study ctDNA in SCCHN and to verify its clinical relevance. Based on current data, we believe that ctDNA has the potential to guide personalized treatment strategies for patients with SCCHN and to hopefully improve their outcome.

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Declaration of Competing Interest

The authors declare that they have no known competing financial

Table 5
Selected studies focusing on HPV cell-free DNA.

Study	N	Sample	Analysis	Technique(s)	Outcome
Cao et al. [86]	40 HPV-positive OPSCC 24 HPV-negative SCCHN 10 non-cancer	Plasma Tumor	HPV 16/18 DNA E6 and E7	Plasma: real-time PCR Tumor HPV status confirmed by p16 IHC, HPV 16/18 PCR or HPV ISH	HPV cfDNA detected in 65% of pretreatment plasma samples in HPV-positive OPC and in none of HPV negative SCCHN or non-cancer controls. Serial measurement in 14 patients showed rapid decline in HPV DNA and undetectable levels at RT completion
Ahn et al. [65]	93 OPSCC or unknown primary SCC (81 HPV-positive, 12 HPV-negative)	Tumor Salivary rinses Plasma	HPV 16 DNA E6 and E7	Real-time PCR	Combined saliva and plasma analysis on pretreatment samples (HPV-16 DNA) compared with HPV status of the tumor Sensitivity: 76% (plasma alone: 67.3% and saliva alone: 52.8%) Specificity: 100% Negative predictive value: 42%, Positive predictive value: 100% In a multivariable analysis, positive posttreatment saliva HPV status was associated with a higher risk of recurrence
Dahlstrom et al. [67]	262 newly diagnosed, previously untreated OPSCC	Tumor Pretreatment serum	HPV 16 DNA E6 and E7	Tumor HPV status determined by PCR Serum HPV DNA detection by real-time PCR	Stage IV and high N category had a higher rate of detectable pretreatment serum HPV DNA HPV-positive patients with negative pretreatment HPV DNA serum had a trend of better PFS
Hanna et al. [85]	17 recurrent/metastatic HPV-positive OPSCC	Plasma: baseline sample and up to 5 additional plasma samples at 14–21 days interval	HPV DNA 16/18/31/33/45 E7	ddPCR	Total tumor burden correlated with HPV cfDNA levels Changes in HPV cfDNA levels with a median of 16 days prior to restaging scans confirmed treatment response or progression Median HPV cfDNA levels negatively correlates with survival
Chera et al. (2019) [84]	103 non-metastatic p16-positive OPSCC after definitive CRT 55 healthy volunteers and 60 with non-HPV associated malignancy	Plasma at baseline, weekly during CRT and at follow-up	HPV DNA 16/18/31/33/35 E7	ddPCR	HPV cfDNA testing, Sensitivity: 89% , Specificity: 97% HPV cfDNA Kinetics (n = 67): 19/67 (28%) had favorable clearance profile: 100% of persistent/recurrent regional disease-free survival (RDFS) at 18 months

cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; CRT, chemoradiation; ddPCR, digital droplet PCR; HPV, human papillomavirus; IHC, immunohistochemistry, ISH, in situ hybridization; OPSCC, oropharyngeal squamous cell carcinoma; SCCHN, squamous cell carcinoma of the head and neck; PCR, polymerase chain reaction; squamous cell carcinoma of the head and neck; RT, radiotherapy; PFS, progression-free survival.

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References

[1] http://globocan.iarc.fr/.
[2] https://seer.cancer.gov/csr/1975_2014/.
[3] Gatta G, Botta L, Sanchez MJ, Anderson LA, Pierannunzio D, Licitra L, et al. Prognoses and improvement for head and neck cancers diagnosed in Europe in early 2000s: the EUROCARE-5 population-based study. Eur J Cancer 2015;51(15):2130–43. https://doi.org/10.1016/j.ejca.2015.07.043. [published Online First: Epub Date].
[4] Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008;359(11):1116–27. https://doi.org/10.1056/NEJMoa0802656. [published Online First: Epub Date].
[5] Burtness BHK, Greil R, Soulières D, Tahara M, De Castro Jr G, et al. Phase 3 study of first-line pembrolizumab (P) for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). ESMO congress; 2018. Abstract nr 048.
[6] Phallen J, Sausen M, Adleff V, Leal A, Hruban C, White J, et al. Direct detection of early-stage cancers using circulating tumor DNA. Sci Transl Med 2017;9(403). https://doi.org/10.1126/scitranslmed.aan2415. [published Online First: Epub Date].
[7] Chaudhuri Aadel A, Chabon Jacob J, Lovejoy Alexander F, Newman Aaron M, Stehr Henning, Azad Tej D, et al. Early detection of molecular residual disease in localized lung cancer by circulating tumor DNA profiling. Cancer Discov 2017;7(12):1394–403. https://doi.org/10.1158/2159-8290.CD-17-0716.
[8] Dawson Sarah-Jane, Tsui Dana WY, Murtaza Muhammed, Biggs Heather, Rueda Oscar M, Chin Suet-Feung, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368(13):1199–209. https://doi.org/10.1056/NEJMoa1213261.
[9] Siravegna Giulia, Marsoni Silvia, Siena Salvatore, Bardelli Alberto. Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 2017;14(9):531–48. https://doi.org/10.1038/nrclinonc.2017.14.
[10] Pantel Klaus, Alix-Panabières Catherine. Circulating tumour cells in cancer patients: challenges and perspectives. Trends Mol Med 2010;16(9):398–406. https://doi.org/10.1016/j.molmed.2010.07.001.
[11] Amorim Maria G, Valieris Renan, Drummond Rodrigo D, Pizzi Melissa P, Freitas Vanessa M, Sinigaglia-Coimbra Rita, et al. A total transcriptome profiling method for plasma-derived extracellular vesicles: applications for liquid biopsies. Sci Rep 2017;7(1). https://doi.org/10.1038/s41598-017-14264-5.
[12] Chan KCA, Jiang P, Chan CWM, Sun K, Wong J, Hui EP, et al. Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. Proc Natl Acad Sci 2013;110(47):18761–8. https://doi.org/10.1073/pnas.1313995110.
[13] Kim Yunee, Jeon Jouhyun, Mejia Salvador, Yao Cindy Q, Ignatchenko Vladimir, Nyalwidhe Julius O, et al. Targeted proteomics identifies liquid-biopsy signatures for extracapsular prostate cancer. Nat Commun 2016;7(1). https://doi.org/10.1038/ncomms11906.
[14] Mayers Jared R, Wu Chen, Clish Clary B, Kraft Peter, Torrence Margaret E, Fiske Brian P, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med 2014;20(10):1193–8. https://doi.org/10.1038/nm.3686.
[15] Cristofanilli Massimo, Budd G Thomas, Ellis Matthew J, Stopeck Alison, Matera Jeri, Miller M Craig, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351(8):781–91. https://doi.org/10.1056/NEJMoa040766.
[16] Cristofanilli Massimo, Pierga Jean-Yves, Reuben James, Rademaker Alfred, Davis Andrew A, Peeters Dieter J, et al. The clinical use of circulating tumor cells (CTCs) enumeration for staging of metastatic breast cancer (MBC): international expert consensus paper. Critical Rev Oncol/Hematol 2019;134:39–45. https://doi.org/10.1016/j.critrevonc.2018.12.004.
[17] Negin Benjamin P, Cohen Steven J. Circulating tumor cells in colorectal cancer: past, present, and future challenges. Curr. Treat. Options in Oncol. 2010;11(1–2):1–13. https://doi.org/10.1007/s11864-010-0115-3.
[18] Resel Folkersma L, OlivierGómez C, San José Manso L, Veganzones de Castro S, Galante Romo I, Vidaurreta Lázaro M, et al. Immunomagnetic quantification of circulating tumoral cells in patients with prostate cancer: clinical and pathological correlation. Arch Esp Urol. 2010;63(1):23–31.
[19] The cobas EGFR Mutation Test v2 premarket application. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMA/pma.cfm.
[20] Wu Y-L, Zhou C, Liam C-K, Wu G, Liu X, Zhong Z, et al. First-line erlotinib versus gemcitabine/cisplatin in patients with advanced EGFR mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study. Ann Oncol 2015;26(9):1883–9. https://doi.org/10.1093/annonc/mdv270.
[21] Song Lele, Jia Jia, Peng Xiumei, Xiao Wenhua, Li Yuemin. The performance of the SEPT9 gene methylation assay and a comparison with other CRC screening tests: a meta-analysis. Sci Rep 2017;7(1). https://doi.org/10.1038/s41598-017-03321-8.
[22] Premarket Approval (PMA). https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMA/pma.cfm?id=P130001.
[23] Church Timothy Robert, Wandell Michael, Lofton-Day Catherine, Mongin Steven J, Burger Matthias, Payne Shannon R, et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. Gut 2014;63(2):317–25. https://doi.org/10.1136/gutjnl-2012-304149.
[24] De Mattos-Arruda L, Weigelt B, Cortes J, Won HH, Ng CKY, Nuciforo P, et al. Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. Ann Oncol 2014;25(9):1729–35. https://doi.org/10.1093/annonc/mdu239.
[25] Murtaza Muhammed, Dawson Sarah-Jane, Pogrebniak Katherine, Rueda Oscar M, Provenzano Elena, Grant John, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. Nat Commun 2015;6(1). https://doi.org/10.1038/ncomms9760.
[26] Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61(4):1659–65.
[27] Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci 2005;102(45):16368–73. https://doi.org/10.1073/pnas.0507904102.
[28] Gahan PB, Anker P, Stroun M. Metabolic DNA as the origin of spontaneously released DNA? Ann N Y Acad Sci 2008;1137:7–17. https://doi.org/10.1196/annals.1448.046. [published Online First: Epub Date].
[29] Sozzi Gabriella, Conte Davide, Leon MariaElena, Cirincione Rosalia, Roz Luca, Ratcliffe Cathy, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. JCO 2003;21(21):3902–8. https://doi.org/10.1200/JCO.2003.02.006.
[30] Mazurek Agnieszka M, Rutkowski Tomasz, Fiszer-Kierzkowska Anna, Małusecka Ewa, Składowski Krzysztof. Assessment of the total cfDNA and HPV16/18 detection in plasma samples of head and neck squamous cell carcinoma patients. Oral Oncol 2016;54:36–41. https://doi.org/10.1016/j.oraloncology.2015.12.002.
[31] Swarup V, Rajeswari MR. Circulating (cell-free) nucleic acids–a promising, non-invasive tool for early detection of several human diseases. FEBS Lett 2007;581(5):795–9. https://doi.org/10.1016/j.febslet.2007.01.051. [published Online First: Epub Date].
[32] Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. 224ra24 Sci Transl Med 2014;6(224). https://doi.org/10.1126/scitranslmed.3007094.
[33] Widschwendter Martin, Evans Iona, Jones Allison, Ghazali Shohreh, Reisel Daniel, Ryan Andy, et al. Methylation patterns in serum DNA for early identification of disseminated breast cancer. Genome Med 2017;9(1). https://doi.org/10.1186/s13073-017-0499-9.
[34] Widschwendter Martin, Zikan Michal, Wahl Benjamin, Lempiäinen Harri, Paprotka Tobias, Evans Iona, et al. The potential of circulating tumor DNA methylation analysis for the early detection and management of ovarian cancer. Genome Med 2017;9(1). https://doi.org/10.1186/s13073-017-0500-7.
[35] Cohen Paul A, Flowers Nicola, Tong Stephen, Hannan Natalie, Pertile Mark D, Hui Lisa. Abnormal plasma DNA profiles in early ovarian cancer using a non-invasive prenatal testing platform: implications for cancer screening. BMC Med 2016;14(1). https://doi.org/10.1186/s12916-016-0667-6.
[36] Genovese G, Jaiswal S, Ebert BL, McCarroll SA. Clonal hematopoiesis and blood-cancer risk. N Engl J Med 2015;372(11):1071–2. https://doi.org/10.1056/NEJMc1500684. [published Online First: Epub Date].
[37] Hu Yuebi, Ulrich Bryan C, Supplee Julianna, Kuang Yanan, Lizotte Patrick H, Feeney Nora B, et al. False-positive plasma genotyping due to clonal hematopoiesis. Clin Cancer Res 2018;24(18):4437–43. https://doi.org/10.1158/1078-0432.CCR-18-0143.
[38] Cancer Res 2006;66(13):6871–6. https://doi.org/10.1158/0008-5472.CAN-05-4556.
[39] Martincorena I, Campbell PJ. Somatic mutation in cancer and normal cells. Science 2015;349(6255):1483–9. https://doi.org/10.1126/science:aab4082.
[40] Oxnard GR, Klein EA, Seiden M, Hubbell E, Venn O, Jamshidi A, et al. Simultaneous multi-cancer detection and tissue of origin (TOO) localization using targeted bisulfite sequencing of plasma cell-free DNA (cfDNA). J. Global Oncol. 2019;5(suppl (October 07, 2019)). 44-44.
[41] Mao M, Li S, Ren Q, Zhong G, Li R, Chen Y, et al. Development of a blood-based cancer screening assay with a novel multivariate cancer risk score (MCRS) model by integrating shallow WGS data and plasma protein markers. J. Global Oncol. 2019;5(suppl (October 07, 2019)). 45-45.
[42] Pantel Klaus, Alix-Panabières Catherine. Liquid biopsy and minimal residual disease—latest advances and implications for cure. Nat Rev Clin Oncol 2019;16(7):409–24. https://doi.org/10.1038/s41571-019-0187-3.
[43] Garcia-Murillas Isaac, Schiavon Gaia, Weigelt Britta, Ng Charlotte, Hrebien Sarah, Cutts Rosalind J, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. 302ra133 Sci Transl Med 2015;7(302). https://doi.org/10.1126/scitranslmed.aab0021.
[44] Tie Jeanne, Wang Yuxuan, Tomasetti Cristian, Li Lu, Springer Simeon, Kinde Isaac, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. 346ra92 Sci Transl Med 2016;8(346). https://doi.org/10.1126/scitranslmed.aaf6219.
[45] Douillard J-Y, Ostoros G, Cobo M, Ciuleanu T, McCormack R, Webster A, Milenkova T. First-line gefitinib in Caucasian EGFR mutation-positive NSCLC patients: a phase-IV, open-label, single-arm study. Br J Cancer 2014;110(1):55–62. https://doi.org/10.1038/bjc.2013.721.
[46] Vidal J, Muinelo L, Dalmases A, Jones F, Edelstein D, Iglesias M, Orrillo M, Abalo A,

Rodríguez C, Brozos E, Vidal Y, Candamio S, Vázquez F, Ruiz J, Guix M, Visa L, Sikri V, Albanell J, Bellosillo B, López R, Montagut C. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Ann Oncol 2017;28(6):1325–32. https://doi.org/10.1093/annonc/mdx125.

[47] Diehl Frank, Schmidt Kerstin, Choti Michael A, Romans Katharine, Goodman Steven, Li Meng, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14(9):985–90. https://doi.org/10.1038/nm.1789.

[48] Newman Aaron M, Bratman Scott V, To Jacqueline, Wynne Jacob F, Eclov Neville CW, Modlin Leslie A. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20(5):548–54. https://doi.org/10.1038/nm.3519.

[49] Diaz Jr Luis A, Williams Richard T, Wu Jian, Kinde Isaac, Hecht J Randolph, Berlin Jordan, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486(7404):537–40. https://doi.org/10.1038/nature11219.

[50] Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, Luke JJ, Butaney M, Kirschmeier P, Jackman DM, Janne PA. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res 2014;20(6):1698–705. https://doi.org/10.1158/1078-0432.CCR-13-2482.

[51] Wan Jonathan CM, Massie Charles, Garcia-Corbacho Javier, Mouliere Florent, Brenton James D, Caldas Carlos, Pacey Simon, Baird Richard, Rosenfeld Nitzan. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer 2017;17(4):223–38. https://doi.org/10.1038/nrc.2017.7.

[52] Hindson Benjamin J, Ness Kevin D, Masquelier Donald A, Belgrader Phillip, Heredia Nicholas J, Makarewicz Anthony J, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 2011;83(22):8604–10. https://doi.org/10.1021/ac202028g.

[53] Sanmamed MF, Fernandez-Landazuri S, Rodriguez C, Zarate R, Lozano MD, Zubiri L, et al. Quantitative cell-free circulating BRAFV600E mutation analysis by use of droplet digital PCR in the follow-up of patients with melanoma being treated with BRAF inhibitors. Clin Chem 2015;61(1):297–304. https://doi.org/10.1373/clinchem.2014.230235. [published Online First: Epub Date].

[54] Diehl Frank, Li Meng, He Yiping, Kinzler Kenneth W, Vogelstein Bert, Dressman Devin. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods 2006;3(7):551–9. https://doi.org/10.1038/nmeth898.

[55] Lebofsky R, Decraene C, Bernard V, Kamal M, Blin A, Leroy Q, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol 2015;9(4):783–90. https://doi.org/10.1016/j.molonc.2014.12.003. [published Online First: Epub Date].

[56] Park Gahee, Park Joo Kyung, Shin Seung-Ho, Jeon Hyo-Jeong, Kim Nayoung KD, et al. Characterization of background noise in capture-based targeted sequencing data. Genome Biol 2017;18(1). https://doi.org/10.1186/s13059-017-1275-2.

[57] Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci 2011;108(23):9530–5. https://doi.org/10.1073/pnas.1105422108.

[58] Newman Aaron M, Lovejoy Alexander F, Klass Daniel M, Kurtz David M, Chabon Jacob J, Scherer Florian, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotechnol 2016;34(5):547–55. https://doi.org/10.1038/nbt.3520.

[59] Wyatt AW, Annala M, Aggarwal R, Beja K, Feng F, Youngren J, et al. Concordance of circulating tumor DNA and matched metastatic tissue biopsy in prostate cancer. J Natl Cancer Inst 2017;109(12). https://doi.org/10.1093/jnci/djx118. [published Online First: Epub Date].

[60] Murtaza Muhammed, Dawson Sarah-Jane, Tsui Dana WY, Gale Davina, Forshew Tim, Piskorz Anna M, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497(7447):108–12. https://doi.org/10.1038/nature12065.

[61] Heitzer Ellen, Ulz Peter, Belic Jelena, Gutschi Stefan, Quehenberger Franz, Fischereder Katja, et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 2013;5(4):30. https://doi.org/10.1186/gm434.

[62] Chan KC, Jiang P, Zheng YW, Liao GJ, Sun H, Wong J, et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem 2013;59(1):211–24. https://doi.org/10.1373/clinchem.2012.196014. [published Online First: Epub Date].

[63] Ho AL, Chau N, Bauman J, Bible K, Chintakuntlawar A, Cabanillas ME, et al. Preliminary results from a phase 2 trial of tipifarnib in Squamous Cell Carcinomas (SCCs) with HRAS mutations. Ann Oncol 2018;29(suppl_8):viii372–99. https://doi.org/10.1093/annonc/mdy287 2018.

[64] Galot R, Le Tourneau C, Guigay J, Licitra L, Tinhofer I, Kong A, et al. Personalized biomarker-based treatment strategy for patients with squamous cell carcinoma of the head and neck: EORTC position and approach. Ann Oncol 2018;29(12):2313–27. https://doi.org/10.1093/annonc/mdy452.

[65] Ahn Sun M, Chan Jason YK, Zhang Zhe, Wang Hao, Khan Zubair, Bishop Justin A, et al. Saliva and plasma quantitative polymerase Chain reaction–based detection and surveillance of human papillomavirus–related head and neck cancer. JAMA Otolaryngol Head Neck Surg 2014;140(9):846. https://doi.org/10.1001/jamaoto.2014.1338.

[66] Chuang Alice Y, Chuang Tony C, Chang Steven, Zhou Shaoyu, Begum Shahnaz, Westra William H, et al. Presence of HPV DNA in convalescent salivary rinses is an adverse prognostic marker in head and neck squamous cell carcinoma. Oral Oncol 2008;44(10):915–9. https://doi.org/10.1016/j.oraloncology.2008.01.001.

[67] Dahlstrom Kristina R, Li Guojun, Hussey Caroline S, Vo Jenny T, Wei Qingyi, Zhao Chong, et al. Circulating human papillomavirus DNA as a marker for disease extent and recurrence among patients with oropharyngeal cancer: HPV DNA as Marker for Recurrence in OPC. Cancer 2015;121(19):3455–64. https://doi.org/10.1002/cncr.v121.1910.1002/cncr.29538.

[68] Nawroz-Danish Homaira, Eisenberger Claus F, Yoo George H, Wu Li, Koch Wayne, Black Carri, et al. Microsatellite analysis of serum DNA in patients with head and neck cancer. Int J Cancer 2004;111(1):96–100. https://doi.org/10.1002/(ISSN)1097-021510.1002/ijc.v111:110.1002/ijc.20240.

[69] Hamana K, Uzawa K, Ogawara K, Shiiba M, Bukawa H, Yokoe H, et al. Monitoring of circulating tumour-associated DNA as a prognostic tool for oral squamous cell carcinoma. Br J Cancer 2005;92(12):2181–4. https://doi.org/10.1038/sj.bjc.6602635.

[70] Schrock A, Leisse A, de Vos L, Gevensleben H, Droge F, Franzen A, et al. Free-circulating methylated DNA in blood for diagnosis, staging, prognosis, and monitoring of head and neck squamous cell carcinoma patients: an observational prospective cohort study. Clin Chem 2017;63(7):1288–96. https://doi.org/10.1373/clinchem.2016.270207. [published Online First: Epub Date].

[71] Mydlarz Wojciech K, Hennessey Patrick T, Wang Hao, Carvalho Andre Lopez, Califano Joseph A. Serum biomarkers for detection of head and neck squamous cell carcinoma: serum biomarkers for HNSCC. Head Neck 2016;38(1):9–14. https://doi.org/10.1002/hed.23842.

[72] Schirmer MA, Beck J, Leu M, Oellerich M, Rave-Frank M, Walson PD, et al. Cell-free plasma DNA for disease stratification and prognosis in head and neck cancer. Clin Chem 2018;64(6):959–70. https://doi.org/10.1373/clinchem.2017.285668. [published Online First: Epub Date].

[73] Wang Yuxuan, Springer Simeon, Mulvey Carolyn L, Silliman Natalie, Schaefer Joy, Sausen Mark, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. 293ra104 Sci Transl Med 2015;7(293). https://doi.org/10.1126/scitranslmed.aaa8507.

[74] Perdomo Sandra, Avogbe Patrice H, Foll Matthieu, Abedi-Ardekani Behnoush, Facciolla Violeta Lescher, Anantharaman Devasena, et al. Circulating tumor DNA detection in head and neck cancer: evaluation of two different detection approaches. Oncotarget 2017;8(42). https://doi.org/10.18632/oncotarget.v8i4210.18632/oncotarget.20004.

[75] van Ginkel Joost H, Huibers Manon MH, van Es Robert JJ, de Bree Remco, Willems Stefan M. Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. BMC Cancer 2017;17(1). https://doi.org/10.1186/s12885-017-3424-0.

[76] Egyud Matthew, Sridhar Praveen, Devaiah Anand, Yamada Emiko, Saunders Stefanie, Ståhlberg Anders, et al. Plasma circulating tumor DNA as a potential tool for disease monitoring in head and neck cancer. Head Neck 2019;41(5):1351–8. https://doi.org/10.1002/hed.v41.510.1002/hed.25563.

[77] Fernandez-Cuesta L, Perdomo S, Avogbe PH, Leblay N, Delhomme TM, Gaborieau V, et al. Identification of circulating tumor DNA for the early detection of small-cell lung cancer. EBioMedicine 2016;10:117–23. https://doi.org/10.1016/j.ebiom.2016.06.032. [published Online First: Epub Date].

[78] Krimmel Jeffrey D, Schmitt Michael W, Harrell Maria I, Agnew Kathy J, Kennedy Scott R, Emond Mary J, et al. Ultra-deep sequencing detects ovarian cancer cells in peritoneal fluid and reveals somatic TP53 mutations in noncancerous tissues. PNAS 2016;113(21):6005–10. https://doi.org/10.1073/pnas.1601311113.

[79] Braig Friederike, Voigtlaender Minna, Schieferdecker Aneta, Busch Chia-Jung, Laban Simon, Grob Tobias, et al. Liquid biopsy monitoring uncovers acquired RAS-mediated resistance to cetuximab in a substantial proportion of patients with head and neck squamous cell carcinoma. Oncotarget 2016;7(28). https://doi.org/10.18632/oncotarget.v7i2810.18632/oncotarget.8943.

[80] Schmidt H, Kulasinghe A, Allcock RJN, Tan LY, Mokany E, Kenny L, et al. A pilot study to non-invasively track PIK3CA mutation in head and neck cancer. Diagnostics (Basel) 2018;8(4). https://doi.org/10.3390/diagnostics8040079. [published Online First: Epub Date].

[81] Fostira F, Oikonomopoulou P, Kladi A, Edelstein D, Stieler K, Heim D, 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_5):v449–74. https://doi.org/10.1093/annonc/mdz252.

[82] Galot R, Lummen CVMd, Helaers R, Mendola A, Goebbels R, Caignet X, et al. Liquid biopsy for mutational profiling of locoregional recurrent and/or metastatic squamous cell carcinoma of the head and neck. Annals of Oncology 2019;30(suppl_5):1–2. https://doi.org/10.1093/annonc/mdz252.

[83] Lechner M, Fenton TR. The genomics, epigenomics, and transcriptomics of HPV-associated oropharyngeal cancer–understanding the basis of a rapidly evolving disease. Adv Genet 2016;93:1–56. https://doi.org/10.1016/bs.adgen.2015.12.001.

[84] Chera Bhishamjit S, Kumar Sunil, Beaty Brian T, Marron David, Jefferys Stuart, Green Rebecca, 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(15):4682–90. https://doi.org/10.1158/1078-0432.CCR-19-0211.

[85] Hanna GJ, Supplee JG, Kuang Y, Mahmood U, Lau CJ, Haddad RI, et al. Plasma HPV cell-free DNA monitoring in advanced HPV-associated oropharyngeal cancer. Ann Oncol 2018;29(9):1980–6. https://doi.org/10.1093/annonc/mdy251.

[86] Cao Hongbin, Banh Alice, Kwok Shirley, Shi Xiaoli, Wu Simon, Krakow Trevor, et al. Quantitation of human papillomavirus DNA in plasma of oropharyngeal carcinoma patients. Int J Radiat Oncol Biol Phys 2012;82(3):e351–8. https://doi.org/10.1016/j.ijrobp.2011.05.061.

[87] van Ginkel Joost H, Slieker Fons JB, de Bree Remco, van Es Robert JJ, Van Cann Ellen M, Willems Stefan M. Cell-free nucleic acids in body fluids as biomarkers for the prediction and early detection of recurrent head and neck cancer: a systematic

review of the literature. Oral Oncol 2017;75:8–15. https://doi.org/10.1016/j.oraloncology.2017.10.007.

[88] Oxnard Geoffrey R, Thress Kenneth S, Alden Ryan S, Lawrance Rachael, Paweletz Cloud P, Cantarini Mireille, et al. Association between plasma genotyping and outcomes of treatment with osimertinib (AZD9291) in advanced non-small-cell lung cancer. JCO 2016;34(28):3375–82. https://doi.org/10.1200/JCO.2016.66.7162.

[89] Lavoie Jean-Michel, Vandekerkhove Gillian, Annala Matti, Sundahl Nora, Sano Takeshi, Struss Werner J, et al. Circulating tumor DNA in patients with metastatic urothelial cancer: concordance of genomic findings with matched tissue biopsies. e16036-e16036 JCO 2019;37(15_suppl). https://doi.org/10.1200/JCO.2019.37.15_suppl.e16036.

[90] Ståhlberg Anders, Krzyzanowski Paul M, Jackson Jennifer B, Egyud Matthew, Stein Lincoln, Godfrey Tony E. Simple, multiplexed, PCR-based barcoding of DNA enables sensitive mutation detection in liquid biopsies using sequencing. e105-e105 Nucleic Acids Res 2016;44(11). https://doi.org/10.1093/nar/gkw224.

[91] Schrock Alexa B, Welsh Allison, Chung Jon H, Pavlick Dean, Bernicker Eric H, Creelan Benjamin C, et al. Hybrid capture–based genomic profiling of circulating tumor DNA from patients with advanced non–small cell lung cancer. J Thoracic Oncol 2019;14(2):255–64. https://doi.org/10.1016/j.jtho.2018.10.008.

[92] Tie Jeanne, Cohen Joshua D, Wang Yuxuan, Christie Michael, Simons Koen, Lee Margaret, Wong Rachel, Kosmider Suzanne, Ananda Sumitra, McKendrick Joseph, et al. Circulating tumor DNA analyses as markers of recurrence risk and benefit of adjuvant therapy for stage III colon cancer. JAMA Oncol 2019;5(12):1710. https://doi.org/10.1001/jamaoncol.2019.3616.

[93] Porter Ashleigh, Daniels Gregory A, Patel Sandip Pravin, Sacco Assuntina Gesualda, Banks Kimberly, Cohen Ezra EW. Next generation sequencing of cell free circulating tumor DNA in blood samples of recurrent and metastatic head and neck cancer patients. e17516-e17516 JCO 2017;35(15_suppl). https://doi.org/10.1200/JCO.2017.35.15_suppl.e17516.

[94] Wu Ping, Tang Yaoyun, Gong Yuhua, Tao Xuan, Guan Yanfang, Yang Ling, et al. Tracking tumor-specific mutations in plasma, saliva and para-carcinoma tissues from patients with head and neck squamous cell carcinomas. 6056-6056 JCO 2018;36(15_suppl). https://doi.org/10.1200/JCO.2018.36.15_suppl.6056.

[95] Soulieres D, Licitra L, Mesia R, Remenar E, Li SH, Karpenko A, et al. Molecular alterations and buparlisib efficacy in patients with squamous cell carcinoma of the head and neck: Biomarker Analysis from BERIL-1. Clin Cancer Res 2018;**24**(11):2505-16 doi: 10.1158/1078-0432.CCR-17-2644[published Online First: Epub Date].