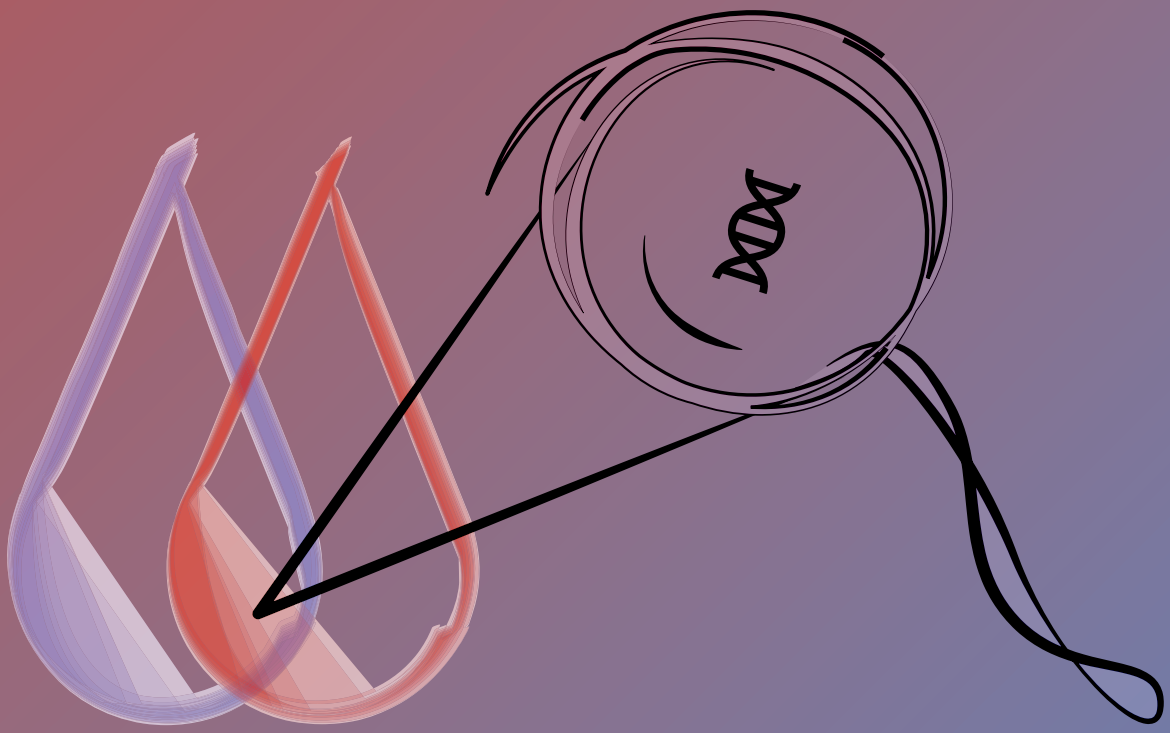
LIQUID BIOPSY IN HEAD AND NECK CANCER

The development of a new diagnostic tool



Joost H. van Ginkel

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The development of a new diagnostic tool

LIQUID BIOPSY IN HOOFD-HALSKANKER

De ontwikkeling van een nieuw diagnosticum
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 3 december 2019 des middags te 12.45 uur

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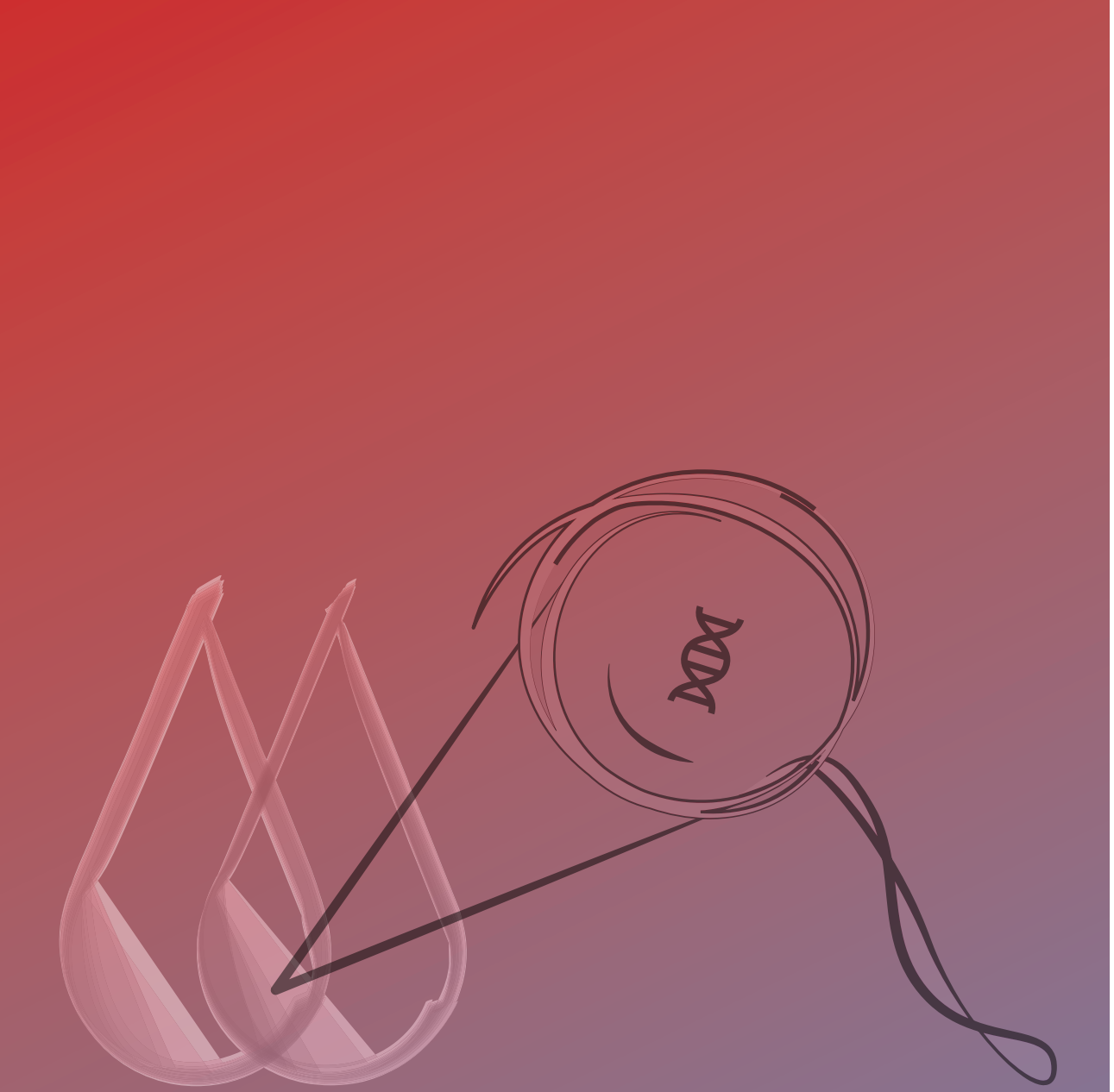

1

General Introduction & Thesis Outline

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GENERAL INTRODUCTION

Head and neck cancer encompasses a group of malignant tumors of the upper aerodigestive tract and adjacent tissues including the oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, nasal cavity and paranasal sinuses (Figure 1). Head and neck squamous cell carcinomas (HNSCC) usually refer to tumors originating from the mucosal linings of the four most common sites: the oral cavity, oropharynx, hypopharynx, and larynx. In comparison, (para)nasal and salivary gland tumors far more often exhibit a wide range of histopathological subtypes other than squamous cell carcinoma, and are thus considered to be different entities. The focus of this thesis will be on HNSCC originating from these four most common sites.

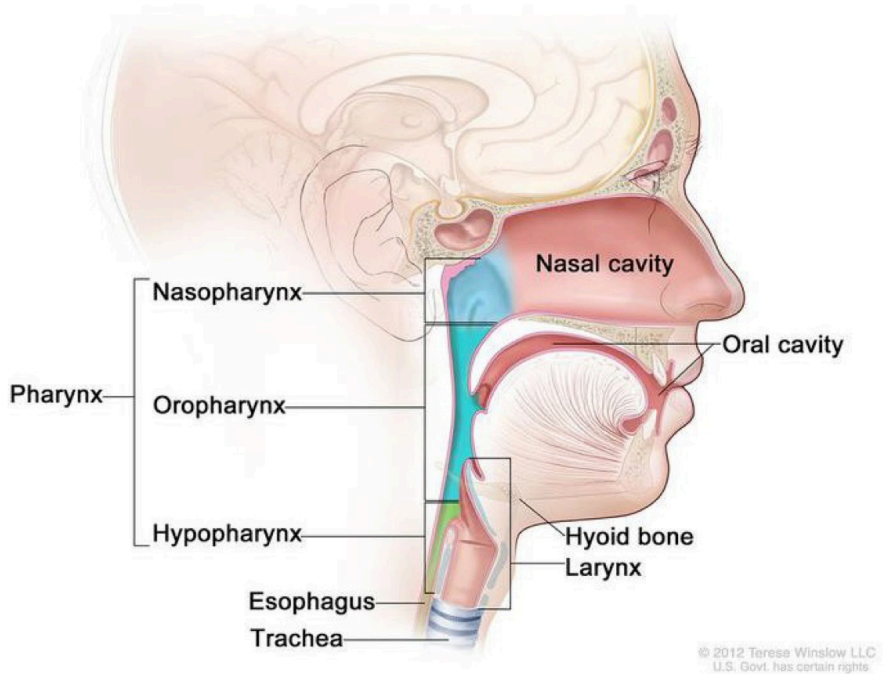


FIGURE 1. Head and neck cancer sites [1].

Etiology

Major risk factors of HNSCC are tobacco smoking, betel nut chewing, alcohol consumption, and human papilloma virus (HPV) infection with HPV types 16 and 18 known as high-risk types [2]. HPV infection-associated tumors are commonly oropharyngeal carcinomas,

which exhibit HPV positivity of tumor tissue in 30.8% of all cases worldwide [3] and currently 48.1% in The Netherlands [4]. In contrast, nasopharyngeal carcinomas are associated with Epstein-Barr virus (EBV) infection in the vast majority of cases and occur most commonly in Southeast Asian countries [5].

Epidemiology

The prognosis of HNSCC is largely based on the disease stage at presentation, particularly the presence of lymph node metastases in the neck and distant metastases [6]. Despite improvements in treatment [7], locoregional recurrence rates after surgery and/or (chemo)radiotherapy range from 25% to 50% depending on tumor location and stage. Current 5-year survival rates in Europe range between 25% and 60% depending on primary tumor site and stage, and improved only marginally in the last 2 decades [7- 9]. This may partly be explained by the poor outcome after treatment of residual or recurrent disease, which in turn may be a consequence of delayed diagnosis due to difficulties in distinguishing locoregional disease from posttreatment effects (e.g. fibrosis, inflammation) on physical examination and imaging, especially during the first months of follow-up [10]. In case of recurrence above the clavicles, salvage surgery generally remains the only curative option [11]. Therefore, a timely diagnosis of locoregional recurrence is crucial to increase the possibility of prompt curative salvage surgery [12]. To date, the standard method for assessment of locoregional control is clinical evaluation, supported by flexible endoscopy and/or imaging. Therefore, posttreatment imaging such as computed tomography (CT), combined with F-18-fluorodeoxyglucose positron emission tomography (FDG-PET/CT), or magnetic resonance imaging (MRI) including diffusion weighted imaging (DWI) as response evaluation or in case of clinical suspicion of recurrence, is performed routinely. However, differentiation between posttreatment effects and tumor recurrence in these imaging modalities is difficult, especially after (chemo)radiotherapy. Although FDG-PET/CT is highly sensitive and specific (87% and 93%) for local recurrence when performed after 3 months, the sensitivity and specificity for regional recurrence is only 79% and 95%, as described in a systematic review and meta-analysis of FDG-PET/CT trials for locoregional surveillance following definitive treatment [13]. This could lead to a considerable amount of false positive cases rendering erroneous therapeutic intervention. As a result, there is a clear need for more specific biomarkers to detect (early) recurrences.

Pathogenesis

The pathogenesis of HNSCC is strongly related to alcohol consumption, tobacco use, and high-risk human papilloma virus (HPV) infection [14]. HPV-negative and HPV-positive tumors have been shown to be (epi)genetically and clinically different entities [15, 16]. In HPV-positive tumors, *PIK3CA* and *PTEN* are found to be the most frequently altered genes [17]. In HPV-

negative tumors, most frequently mutated genes are *TP53*, *CDKN2A*, and *NOTCH* [18, 19]. Moreover, TP53 mutations appear to play an important role in the early onset of HNSCCs [20-22]. Subsequent loss of heterozygosity (LOH) leads to loss of the non-mutated gene [23, 24] and newly formed clonal expansions [25, 26]. Frequently and early occurring somatic *TP53* mutations during HNSCC carcinogenesis are highly present in clonal progenitor cells, and could be used as a biomarker to detect the presence of primary and/or metastatic tumor, assuming these mutations to be globally inherited by subclonal outgrowths of primary tumors.

Liquid Biopsy in Head and Neck Cancer

Various DNA sequencing techniques, such as next-generation sequencing (NGS) and Sanger sequencing, are currently applied in clinical practice using tissue biopsies in order to guide cancer therapy [27]. However, its invasive character accompanied with high patient burden and risk of complications are major drawbacks of tissue biopsy [28]. Moreover, HNSCC locoregional (micro)metastases are often too small to be detected on imaging for guided tissue biopsy sampling. Consequently, although tissue biopsies can deliver useful information about the primary tumor genetic profile, there are limitations to its use as a method for monitoring posttreatment surveillance of locoregional and distant disease.

Body fluids such as blood and saliva are other sources for DNA retrieval in an attempt to monitor tumor status, also called 'liquid biopsy'. First identified in 1948, blood of healthy individuals contains genetic material in the form of cell free DNA (cfDNA) [29]. Additionally, in patients with a malignant tumor, blood can contain both circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) [30-32], probably being released into the bloodstream by (metastatic) tumor cells following apoptosis and necrosis, or by active release of living cells [33]. CfDNA has already been studied clinically in prenatal testing, transplant patients, and trauma patients [34, 35], while detection of ctDNA has been suggested as a potential biomarker for various cancers including HNSCC [36-40].

The clinical applications of ctDNA in cancer treatment are roughly divided into two categories: characterizing tumor genetics and quantitation of ctDNA representing tumor burden [41]. Applications based on ctDNA for characterizing tumor genetics can be used as a tool to guide targeted drug therapy, particularly in metastatic disease, and as an alternative to conventional tissue biopsy in cases of absolute or relative contraindications to tissue biopsy. The second category of applications could be used as a disease monitoring tool, as there appears to be a relation between ctDNA load and tumor burden [42]. Diehl et al. evidenced that in 15 of 16 patients who underwent curative colorectal cancer surgery and developed disease recurrence, plasma ctDNA with tumor specific genetic alterations was still detectable after surgery [43]. In another pilot study the possibility of prognostication and monitoring of oral SCC was assessed by analyzing serum-isolated DNA of 64 patients

using microsatellite markers to detect allelic imbalances. In more than 50% of patients allelic imbalances were identified in serum DNA corresponding with tumor DNA. In turn, a correlation was found between allelic imbalances in serum DNA and tumor stage [40]. Although the use of microsatellites has been subject of debate [44], these results are promising. More recently, Bettegowda et al. reported detectable ctDNA levels in 55% of 223 patients with localized tumors of varying origin (i.e. pancreatic, ovarian, colorectal, bladder, gastro-esophageal, breast, melanoma, hepatocellular, and head and neck). Also, a direct proportional correlation was found between the fraction of patients with detectable ctDNA levels and tumor stage [45]. Regarding posttreatment tumor monitoring, liquid biopsy could be an interesting tool in the follow-up of HNSCC patients.

Firstly, performing Next Generation Sequencing (NGS) on the primary tumor is necessary to determine patient specific genetic mutations in the primary tumor. Up to date, most NGS testing on HNSCCs is performed within the context of research and based on whole-exome sequencing (WES) requiring time-consuming data analysis [46]. By using HNSCC-specific gene panels instead, a more targeted approach of NGS is possible and focuses solely on genes of interest. This allows for a faster and more sensitive sequencing method [27, 47], which is clinically better applicable. Subsequently, based on these NGS results, early driver gene mutations can be selected and used as templates to create mutation specific assays. By collecting liquid biopsies from the patient at different time points before and during/after (chemo)radiotherapy or primary tumor resection, ctDNA can be isolated and used as a biomarker. With mutation specific assays tumor specific mutations can be detected in ctDNA. Due to the relatively short half-life of ctDNA of approximately 2 hours, tumor changes can be evaluated in hours rather than weeks to months [28]. This allows to monitor disease progression or regression very closely and the early detection of tumor recurrence or metastases after initial treatment with curative intent. This manner of personalized cancer management has the potential to prevent overtreatment and insufficient treatment with the possibility to avoid complications of invasive diagnostic techniques and disease progression respectively. A structured workflow for the use of liquid biopsy in clinical practice is proposed in figure 2.

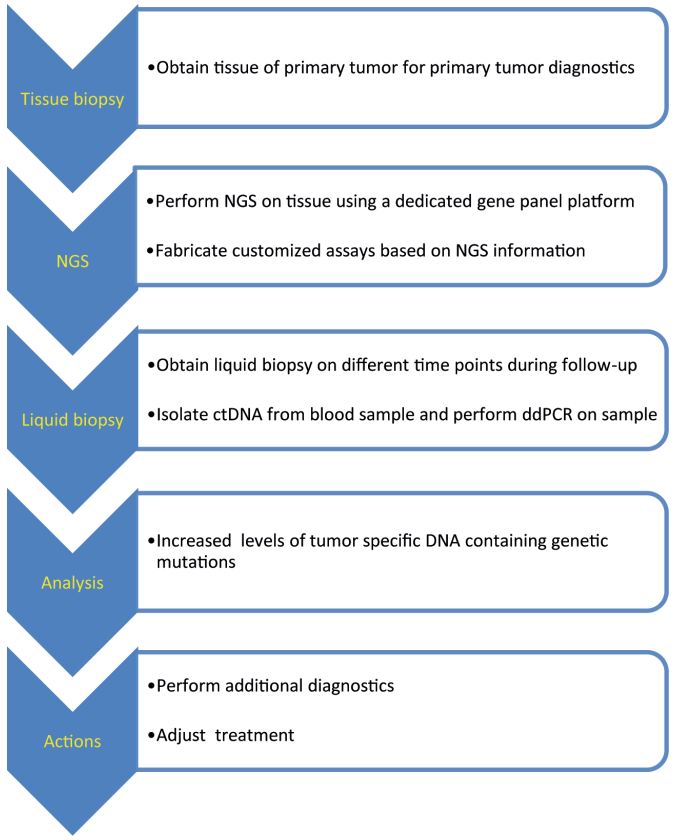


FIGURE 2. Proposed workflow for the practice of liquid biopsy in HNSCC

Although the earlier mentioned data underline the emerging evidence that monitoring genetic alterations in ctDNA is a promising tool to monitor disease recurrence and stage**,** several important technical and biological obstacles have yet to be addressed in order to be able to implement this diagnostic tool in clinical practice [41]. First, high analytic sensitivity is necessary to reliably isolate and detect ctDNA when present in the blood. Secondly, a high proportion of patients should carry detectable amounts of ctDNA, because absolute ctDNA levels vary within each subpopulation [45]. Thirdly, a low signal- to-noise ratio due to the presence of high levels of cfDNA (i.e. wildtype DNA) could interfere with the detection of target ctDNA. Lastly, tumor heterogeneity remains an important challenge. Clonal expansions can arise within the primary tumor, carrying a different mutational profile. During tissue biopsy for initial genetic tumor profiling, a (rare) subclonal mutation could be selected as the target mutation for detection in blood. This could possibly lead to inaccurate representation of tumor burden due to inherently low levels of targeted ctDNA [40].

Droplet digital PCR

A novel tool in genetic diagnostics is droplet digital PCR (ddPCR). Combined with the mutation specific primers, samples will be processed with oil to create 20,000 water-in-oil droplets containing the DNA molecules. The readout of the droplets is an end-point PCR based on Poisson statistics, suggesting that target DNA molecules are distributed randomly over the droplets. After actual PCR has been conducted, some reactions contain target copies while others do not. The yield will be a read-out as positive end- point and negative end-point respectively [48]. This enables the absolute quantitation of nucleic acids in a sample, which will provide great precision due to the partition of the sample by 20,000 fold. Thus, this will facilitate the detection of rare targets of interest. Furthermore, accurate quantification of targets inside the droplets enables the reduction of error rates due to normal PCR efficiency bias. This could accommodate the earlier mentioned issue of a low signal-to-noise ratio in ctDNA detection. Several studies already showed promising results concerning the accuracy of ddPCR during prospective posttreatment monitoring of patients with various types of cancer [49-51]. Although similar prospective data from ddPCR are currently lacking for HNSCC patients, the advantages of ddPCR may efficiently contribute to the diagnostic process in posttreatment monitoring of these patients.

AIMS AND OUTLINE OF THIS THESIS

The aim of this thesis is to assess the role of liquid biopsy in the locoregional surveillance of HNSCC patients following curative treatment and to determine if ddPCR is an appropriate technique to use for the detection of tumor specific alterations in ctDNA. In order to do so, we conducted this research in a stepwise manner by roughly dividing it into a preclinical and a clinical phase. Firstly, several studies were carried out aiming to determine which genetic targets might be of interest as (diagnostic) biomarkers and to assess if ddPCR is feasible for the proposed aim. For this research, we will analyze an extensive database of NGS results with accompanying clinical data, as well as several archived plasma samples from HNSCC patients, respectively. Subsequently, in a prospective longitudinal pilot study analyses of blood samples of HNSCC patients at different time points (before treatment and after treatment) will be carried out. This study will attempt to determine the feasibility of using ddPCR and other minimally invasive and sensitive techniques for disease surveillance after treatment of HNSCC patients.

With the introduction and development of NGS (and other DNA sequencing techniques) over recent decades, large improvements have been made in understanding tumor genetics. Previous studies have shown that *TP53* is highly prone to loss of heterozygosity, leading to

the early occurrence of inactivating non-hotspot mutations of *TP53* [20, 22, 25]. Subclonal cells branched off from the primary tumor either proliferate towards metastases or develop into local recurrences. These clonal expansions are likely to contain the early onset mutations found initially in the primary tumor [21]. In **Chapter 2**, a large dataset of NGS sequencing results will be analyzed using dedicated HNSCC gene panels to explore the mutational status of TP53 and other genes in HNSCCs, in order to identify potential diagnostic biomarkers. All included samples are formalin fixed paraffin embedded (FFPE) biopsy specimens acquired and analyzed previously at the University Medical Center Utrecht (UMCU) for clinical purpose. To outline the use of liquid biopsy in HNSCC patients in current literature a systematic review will be performed in **Chapter 3**. All published studies investigating putative molecular targets for post treatment disease monitoring and/or the prognostic outcome of disease by the detection of cell-free nucleic acids in blood or saliva will be systematically identified and qualified. Subsequently, the technical feasibility of ddPCR on archived pretreatment plasma samples from 6 stage II-IV HNSCC patients will be explored in **Chapter 4**. The ddPCR workflow is extensively tested and refined in a separately performed methodological study in **Chapter 5**. The need for better methods of earlier detection of recurrent HNSCC is urgent, considering that HNSCC 5-year survival rates improved only marginally over recent decades [7, 52]. A prospective study will be performed investigating molecular biomarkers of interest and technical feasibility of their detection in liquid biopsies of clinical patients. In **Chapter 6**, preliminary ddPCR analysis results are shown of both plasma and saliva samples from five HNSCC patients prospectively collected before and during (chemo)radiotherapy.

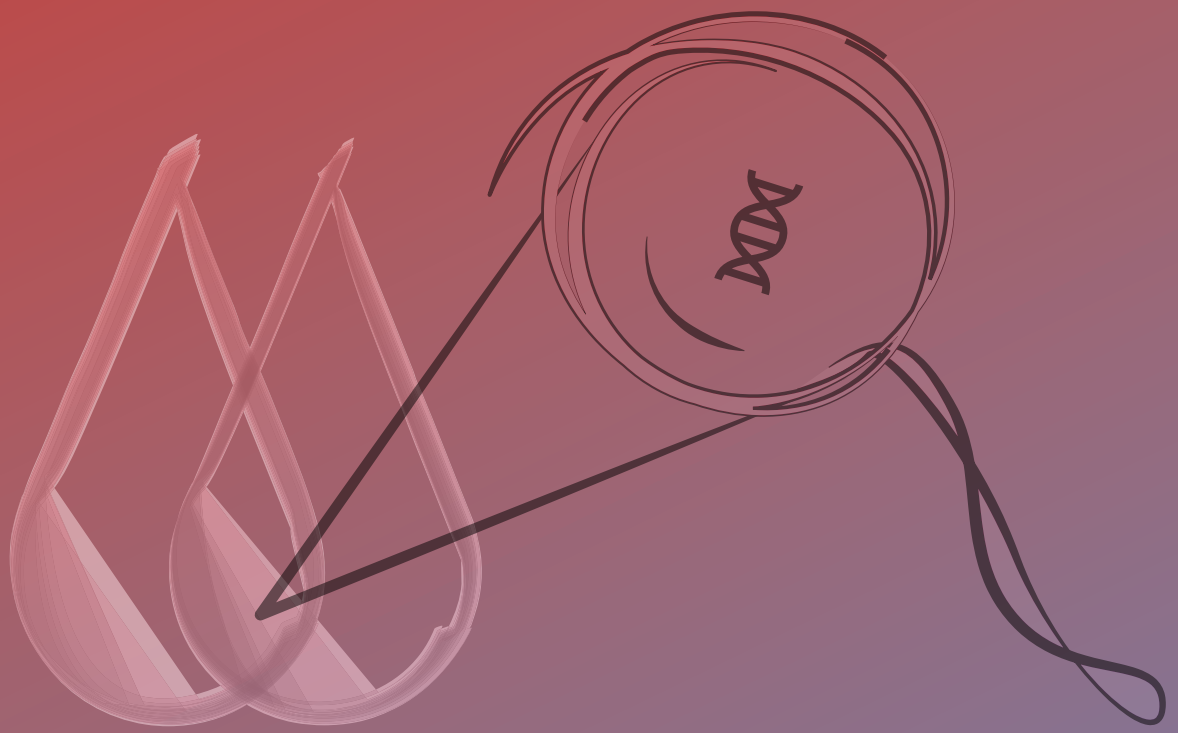
REFERENCES

1. Winslow T. Anatomy of the pharynx. Terese Winslow LLC, Medical And Scientific Illustration; 2012.
2. Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nature reviews Cancer. 2018;18:269-82.
3. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. Lancet Glob Health. 2016;4:e609-16.
4. Rietbergen MM, van Bokhoven A, Lissenberg-Witte BI, Heideman DAM, Leemans CR, Brakenhoff RH, et al. Epidemiologic associations of HPV-positive oropharyngeal cancer and (pre)cancerous cervical lesions. International journal of cancer Journal international du cancer. 2018;143:283-8.
5. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. CA Cancer J Clin. 2017;67:51-64.
6. Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, et al. Head and Neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. CA Cancer J Clin. 2017;67:122-37.
7. Braakhuis BJ, Leemans CR, Visser O. Incidence and survival trends of head and neck squamous cell carcinoma in the Netherlands between 1989 and 2011. Oral oncology. 2014;50:670-5.
8. De Angelis R, Sant M, Coleman MP, Francisci S, Baili P, Pierannunzio D, et al. Cancer survival in Europe 1999-2007 by country and age: results of EUROCARE--5-a population- based study. The Lancet Oncology. 2014;15:23-34.
9. 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;51:2130-43.
10. Leung AS, Rath TJ, Hughes MA, Kim S, Branstetter BFt. Optimal timing of first posttreatment FDG PET/CT in head and neck squamous cell carcinoma. Head & neck. 2016;38 Suppl 1:E853-8.
11. Ho AS, Kraus DH, Ganly I, Lee NY, Shah JP, Morris LG. Decision making in the management of recurrent head and neck cancer. Head & neck. 2014;36:144-51.
12. de Bree R, van der Putten L, Brouwer J, Castelijns JA, Hoekstra OS, Leemans CR. Detection of locoregional recurrent head and neck cancer after (chemo)radiotherapy using modern imaging. Oral oncology. 2009;45:386-93.
13. ytriw AA, Yu E, Waldron J, Lu L, Fazelzad R, et al. (18) F-FDG PET/CT for locoregional surveillance following definitive treatment of head and neck cancer: A meta-analysis of reported studies. Head & neck. 2019;41:551-61.
14. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nature reviews Cancer. 2011;11:9-22.
15. van Kempen PM, Noorlag R, Braunius WW, Stegeman I, Willems SM, Grolman W. Differences in methylation profiles between HPV-positive and HPV-negative oropharynx squamous cell carcinoma: a systematic review. Epigenetics. 2014;9:194-203.

16. O'Rorke MA, Ellison MV, Murray LJ, Moran M, James J, Anderson LA. Human papillomavirus related head and neck cancer survival: a systematic review and meta- analysis. Oral oncology. 2012;48:1191-201.
17. Chung CH, Guthrie VB, Masica DL, Tokheim C, Kang H, Richmon J, et al. Genomic alterations in head and neck squamous cell carcinoma determined by cancer gene- targeted sequencing. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26:1216-23.
18. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333:1154-7.
19. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333:1157-60.
20. Nees M, Homann N, Discher H, Andl T, Enders C, Herold-Mende C, et al. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. Cancer research. 1993;53:4189-96.
21. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer research. 2003;63:1727-30.
22. Braakhuis BJ, Leemans CR, Brakenhoff RH. A genetic progression model of oral cancer: current evidence and clinical implications. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2004;33:317-22.
23. Graveland AP, Golusinski PJ, Buijze M, Douma R, Sons N, Kuik DJ, et al. Loss of heterozygosity at 9p and p53 immunopositivity in surgical margins predict local relapse in head and neck squamous cell carcinoma. International Journal of Cancer. 2011;128:1852-9.
24. Graveland AP, Bremmer JF, de Maaker M, Brink A, Cobussen P, Zwart M, et al. Molecular screening of oral precancer. Oral oncology. 2013;49:1129-35.
25. Califano J, vanderRiet P, Westra W, Nawroz H, Clayman G, Piantadosi S, et al. Genetic progression model for head and neck cancer: Implications for field cancerization. Cancer research. 1996;56:2488-92.
26. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57-70.
27. Gagan J, Van Allen EM. Next-generation sequencing to guide cancer therapy. Genome medicine. 2015;7:80.
28. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014;32:579-86.
29. Mandel P, Metais P. *Les Acides Nucleiques Du Plasma Sanguin Chez Lhomme. Cr Soc Biol. 1948;142:241-3.
30. Alix-Panabieres C, Schwarzenbach H, Pantel K. Circulating tumor cells and circulating tumor DNA. Annu Rev Med. 2012;63:199-215.
31. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nature reviews Cancer. 2008;8:329- 40.
32. Wollenberg B, Walz A, Kolbow K, Pauli C, Chaubal S, Andratschke M. Clinical relevance of circulating tumour cells in the bone marrow of patients with SCCHN. Onkologie. 2004;27:358-62.
33. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nature reviews Clinical oncology. 2013;10:472-84.
34. Lui YY, Dennis YM. Circulating DNA in plasma and serum: biology, preanalytical issues and diagnostic applications. Clin Chem Lab Med. 2002;40:962-8.
35. Lo YMD, Rainer TH, Chan LYS, Hjelm NM, Cocks RA. Plasma DNA as a prognostic marker in trauma patients. Clin Chem. 2000;46:319-23.
36. da Silva BF, Gurgel APAD, Neto MADL, de Azevedo DA, de Freitas AC, Neto JDS, et al. Circulating cell-free DNA in serum as a biomarker of colorectal cancer. Journal of clinical pathology. 2013;66:775-8.
37. Hao TB, Shi W, Shen XJ, Qi J, Wu XH, Wu Y, et al. Circulating cell-free DNA in serum as a biomarker for diagnosis and prognostic prediction of colorectal cancer. British journal of cancer. 2014;111:1482-9.
38. Umetani N, Giuliano AE, Hiramatsu SH, Amersi F, Nakagawa T, Martino S, et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006;24:4270-6.
39. Qiu MT, Wang J, Xu YT, Ding XX, Li M, Jiang F, et al. Circulating Tumor DNA Is Effective for the Detection of EGFR Mutation in Non-Small Cell Lung Cancer: A Meta- analysis. Cancer Epidem Biomar. 2015;24:206-12.
40. 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. British journal of cancer. 2005;92:2181-4.
41. Ignatiadis M, Lee M, Jeffrey SS. Circulating Tumor Cells and Circulating Tumor DNA: Challenges and Opportunities on the Path to Clinical Utility. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21:4786-800.
42. Thierry AR, Mouliere F, Gongora C, Ollier J, Robert B, Ychou M, et al. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. Nucleic Acids Res. 2010;38:6159-75.
43. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nature Medicine. 2008;14:985-90.
44. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer--a survey. Biochimica et biophysica acta. 2007;1775:181-232.
45. 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. Science translational medicine. 2014;6:224ra24.
46. Tabatabaeifar S, Kruse TA, Thomassen M, Larsen MJ, Sorensen JA. Use of next generation sequencing in head and neck squamous cell carcinomas: A review. Oral oncology. 2014;50:1035-40.
47. Jones S, Anagnostou V, Lytle K, Parpart-Li S, Nesselbush M, Riley DR, et al. Personalized genomic analyses for cancer mutation discovery and interpretation. Science translational medicine. 2015;7:283ra53.
48. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical chemistry. 2011;83:8604-10.
49. Olsson E, Winter C, George A, Chen Y, Howlin J, Tang MH, et al. Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. EMBO molecular medicine. 2015;7:1034-47.
50. Reinert T, Scholer LV, Thomsen R, Tobiasen H, Vang S, Nordentoft I, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2015.
51. Yanagita M, Redig AJ, Paweletz CP, Dahlberg SE, O'Connell A, Feeney N, et al. A prospective evaluation of

circulating tumor cells and cell-free DNA in EGFR mutant non- small cell lung cancer patients treated with erlotinib on a phase II trial. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016.

52. Simpson MC, Massa ST, Boakye EA, Antisdel JL, Stamatakis KA, Varvares MA, et al. Primary Cancer vs Competing Causes of Death in Survivors of Head and Neck Cancer. JAMA Oncol. 2018;4:257-9.



2

Targeted sequencing reveals *TP53* as a potential diagnostic biomarker in the post treatment surveillance of head and neck cancer

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ABSTRACT

Head and neck squamous cell carcinomas (HNSCC) form a large heterogeneous group of tumors and have a relatively poor outcome in advanced cases. Revealing the underlying genetic mutations in HNSCC facilitates the development of diagnostic biomarkers, which might lead to improved diagnosis and post treatment surveillance. We retrospectively analyzed mutational hotspots using targeted next-generation sequencing (NGS) of 239 HNSCC tumor samples in order to examine the mutational profile of HNSCC. Furthermore, we assessed prevalence, co-occurrence, and synonymy of gene mutations in (matched) tumor samples. *TP53* was found mutated the most frequent with mutation rates of up to 83% in all tumors, compared to mutation rates of between 5 and 23% of *CDKN2A*, *PIK3CA*, *HRAS*, *CDK4*, *FBXW7* and *RB1*. Mutational co-occurrence predominantly existed between *TP53* and *PIK3CA*, *TP53* and *CDKN2A*, and *HRAS* and *PIK3CA*. Mutational synonymy between primary tumor and associated metastasis and recurrence was present in respectively 88% and 89%. *TP53* mutations were concordantly mutated in 95% of metastases and in 91% of recurrences. This indicates *TP53* mutations to be highly prevalent and concordant in primary tumors and associated locoregional metastases and recurrences. In turn, this provides ground for further investigating the use of *TP53* mutations as diagnostic biomarkers in HNSCC patients.

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) originate from various anatomic sites in the upper aerodigestive epithelium, i.e. the oral and (para)nasal cavities, pharynx and larynx. HNSCC is the sixth most common cancer worldwide with an estimated incidence of 4.8% of all malignancies in the entire body [1]. Etiologically, these tumors roughly fall into two main distinct groups: tumors induced either by tobacco smoking or chewing (e.g. betelnut) and alcohol abuse, or by viral infection with Human Papilloma Virus (HPV) or Epstein Barr Virus (EBV). Alcohol and/or tobacco induced HNSCCs are strongly associated with somatic mutations in tumor suppressor genes (TSG) such as *TP53, CDNK2A, PTEN*, and oncogenes (OG) such as *HRAS* and *PIK3CA* [2-5]. *TP53* inactivating HPV oncoproteins E6 and E7 are the main cancer initiators in an increasing number of oropharyngeal squamous cell carcinoma (OPSCC) cases [6]. The overall survival of advanced cases still remains poor. This is especially true for HPV-negative tumors as compared to HPV-positive tumors [7, 8]. Furthermore, the mutational profile of HNSCC appears to significantly affect its disease course and prognosis [9-12]. Although disease outcome of HNSCC depends on multiple levels of disease processes (e.g. pathogenesis, molecular characteristics, and TNM-stage), estimation of its prognosis is still largely based on the tumor stage at clinical presentation and relapse after initial treatment. Furthermore, possibility of successful salvage treatment is largely dependent on early detection and the extent of the locoregional disease [13-16]. This underlines the need to explore new possibilities for improved diagnostics on a molecular level. The use of diagnostic biomarkers could enable detection of tumor specific mutations in order to monitor tumor response after treatment with curative intent. Ultimately, this might improve treatment outcome of HNSCC patients, while avoiding unnecessary (over)treatment and its associated morbidity and accompanying hindrance to the patient.

Technological advances over recent decades have improved the understanding of tumor genetics. Consequently, targeted profiling of tumor genetics is gradually shifting from an experimental setting towards its use in routine clinical practice in fields such as breast and lung oncology [17]. Although no common ground exists yet for the use of biomarkers in clinical decision making for HNSCC patients, evidence for future use is arising [18-20]. As previous studies have shown, *TP53* is highly prone to loss of heterozygosity. This leads to the presence of inactivating non-hotspot mutations of *TP53* that occur early in HNSCC carcinogenesis [21-24]. Subsequently, subclonal cells from the primary tumor either proliferate towards metastases or locally reside after treatment and develop into recurrences. These clonal expansions are likely to contain the early onset mutations found initially in the primary tumor [25-27]. By using dedicated and clinically accessible gene panels based on NGS, these mutations can reliably be detected and selected as targets. Circulating tumor DNA (ctDNA) released by clonal expansion cells contain these targets and could be quantified using

minimally invasive blood samples, as there appears to be a relation between ctDNA plasma concentrations and tumor burden [28-31]. However, the significance of ctDNA in correlation with actual tumor burden and/or tumor growth still needs to be proven for HNSCC patients. This requires research on the identification of early driver gene mutations of HNSCC tumors. Therefore, we retrospectively analyzed a large dataset of sequenced HNSCCs, to map their mutational profile and to explore TP53 and possible other genes as potential diagnostic biomarkers in HNSCC.

RESULTS

Patient and Tumor Characteristics

A total of 110 patients accounted for 239 tumor samples that remained for analysis. Eighty (73%) patients were male. Of all patients, 76 (69%) had a history of smoking tobacco and 67 (61%) had a history of alcohol consumption. Eleven (10%) patients never used tobacco or alcohol. For 18 patients, either or both tobacco smoking and alcohol use was unknown. Table 1 summarizes patient and tumor characteristics of our study group. Of all 239 tumor samples, 148 (62%) were primary site squamous cell carcinomas. Of the primary tumor samples, 53 (36%) originated from the oral cavity, 37 (25%) from the oropharynx, 16 (11%) the hypopharynx, and 28 (19%) from the larynx. Fourteen (9%) primary tumors originated from miscellaneous sites (i.e. nasopharynx and upper esophagus and trachea). Of the 37 OPSCCs, 33 were tested for HPV-status. Only four (12%) samples proved HPV-positive. The remaining 91 out of 239 tumor samples comprised of 29 (12%) recurrences and 62 (26%) metastases. The latter could be subdivided into 38 (62%) nodal metastases and 23 (38%) distant metastases in the lung, liver, bones, or skin.

Mutational analysis

Sequencing was based on Cancer Hotspot Panel v2 (CHPv2) for 160 tumor samples, OncoAmp Panel v2 (OAPv2) was used for 40 samples, and Cancer Hotspot Panel v2+ (CHPv2+) was used for sequencing of 11 samples (table 2). Additional Sanger sequencing was performed in 28 cases, in which NGS failed due to insufficient DNA quantity. NGS of the exons that are included in the three different gene panels (as described in our method section) yielded mutations in 26 different genes. No mutations were detected in *ABL1, MYD88, NOTCH1, AKT1, ARAF, GNAS, GNA11, NRAS, PDGFRA, CALR, CDH1, IDH1, PTPN11, RET, SMO, SRC, STK11, VHL, MLH1, MPL, JAK3, JAK2, IDH2, CRAF, CSF1R, CTNNB1, and EZH2*.

TABLE 1. Patient and Tumor Characteristics

Patients	110
Tumor samples	239
Mean age, years (range)	66 (45-90)
Sex	**n (%)**
Male	80 (73)
Female	30 (27)
Smoking history	**n (%)**
Yes	76 (69)
No	20 (18)
Unknown	14 (13)
Alcohol use	**n (%)**
Former/active	67 (61)
Never	32 (29)
Unknown	11 (10)
Clinical stage*	**n (%)**
T1-2	100 (70)
T3-4	44
N0	94 (67)
N1-2	50
Unknown	5
Primary tumor sites	**n (%)**
Oral cavity	53 (36)
Oropharynx	37 (25)
Hypopharynx	16 (11)
Larynx	28 (19)
Miscellaneous	14 (9)
Tumor subtype	**n (%)**
Primary	148 (62)
Recurrence	29 (12)
Metastasis	62 (26)
HPV status**	**n (%)**
Positive	4 (12)
Negative	29 (88)

* Included all primary and secondary primary tumors

** All positive tumors were OPSCCs

TP53 had the highest mutation rates in recurrences (83%), metastases (82%) and primary tumor samples (76%). These rates compared to mutation rates of *CDKN2A*, *PIK3CA*, *HRAS*, *CDK4*, *FBXW7* and *RB1* of between 5 and 23% (Figure 1A).

TABLE 2. Used gene panels for sequencing of tumor samples

	CHPv2	CHPv2+	OAv2	Sanger	Total
Primary tumor	99	9	22	18	148
OPSCC	*24*	*3*	*4*	*6*	*37*
OSCC	*32*	*6*	*8*	*7*	*53*
HSCC	*11*	-	*4*	*1*	*16*
LSCC	*21*	-	*4*	*3*	*28*
Misc	*11*	-	*2*	*1*	*14*
Recurrence	19	2	7	1	29
Metastasis	42	-	11	9	62
Total	160	11	40	28	239

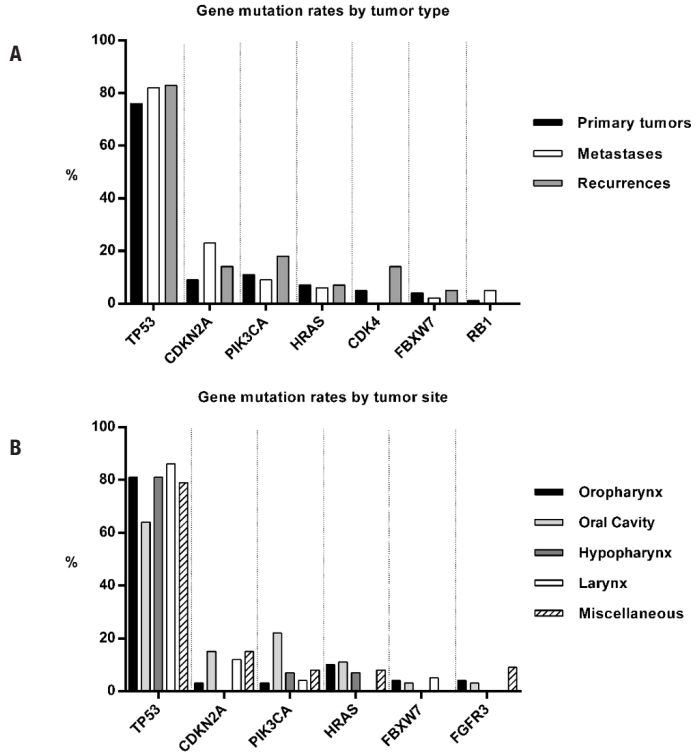

FIGURE 1. Summary of gene mutations in HNSCC samples. Bar charts showing mutation rates (%) of genes in all tumor samples ordered by tumor type (A), and in 148 primary tumor samples ordered by HNSCC sites (B).

Furthermore, *TP53* was found mutated most frequently in OPSCC (81%), OSCCs (64%), HPSCCs (81%), LSCC (86%), and in miscellaneous tumors (79%). On average, other

frequently mutated genes in HNSCC sites were *PIK3CA* (11%), *CDKN2A* (10%), *HRAS* (8%), *FGFR3* (3%) and *FBXW7* (3%) (Figure 1B). In the 130 successfully sequenced primary tumor samples, no mutations were detected in 21 (16%) samples. In one of the HPV-positive tumor samples, a single *TP53* mutation (c.225-35G>C) was found. In the other HPV-positive samples, no mutations were detected. Full range of mutated genes with prevalence rates for all subgroups is provided in supplementary tables S1 and S2. The 92 patients with a history of alcohol use and/or smoking accounted for 121 primary tumor samples in total. *TP53* was sequenced in all 121 samples. *CDKN2A*, *PIK3CA*, and *HRAS* were sequenced in 110 of 121 samples (Table 3).

TABLE 3. Prevalence of gene mutations in alcohol and/or smoking related tumor samples

Mutant gene	Smoking/alcohol		Non-smoking/ alcohol		Smoking		Alcohol		Total	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
TP53	55/69	(80)	9/16	(56)	22/25	(88)	9/11	(82)	96/121	(79)
CDKN2A	2/60	(4)	1/14	(7)	6/25	(24)	1/10	(10)	11/110	(10)
PIK3CA	2/60	(4)	6/14	(43)	3/25	(12)	0/10	-	11/110	(10)
HRAS	1/60	(2)	3/14	(21)	2/25	(8)	2/10	(20)	8/110	(7)

In all tumor samples of patients with or without a history of smoking and alcohol use, highest mutation rates were found in *TP53*, *CDKN2A*, *PIK3CA*, and *HRAS*. Mutation rates for *TP53* were between 56 and 88%, for *CDKN2A* between 4 and 24%, for *PIK3CA* between 0 and 43%, and for *HRAS* between 2 and 21%. Overall, tumors exclusively related to a history of smoking had the highest mutation rates compared to the other subgroups (Figure 2A-D).

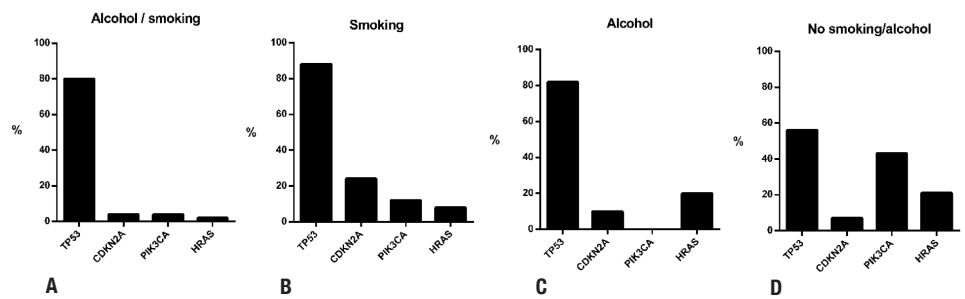

FIGURE 2. Summary of gene mutations in HNSCC samples of patients with or without a history of smoking and/or alcohol use. Bar charts showing mutation rates of primary tumor samples related to a history of smoking and alcohol use (A), exclusively smoking (B), exclusively alcohol use (C), and samples not related to a history of smoking and alcohol use (D).

Mutational co-occurrence

All tumor samples accounted for 171 mutational co-occurrences of two genes within one tumor sample. Co-occurrences were mostly found between *TP53* and *CDKN2A* (16%), *TP53* and *PIK3CA* (9%), *TP53* and *HRAS* (5%), *PIK3CA* and *HRAS* (4%), and *PIK3CA* and *CDKN2A* (3%), as shown in figure 3 and supplementary table S3.

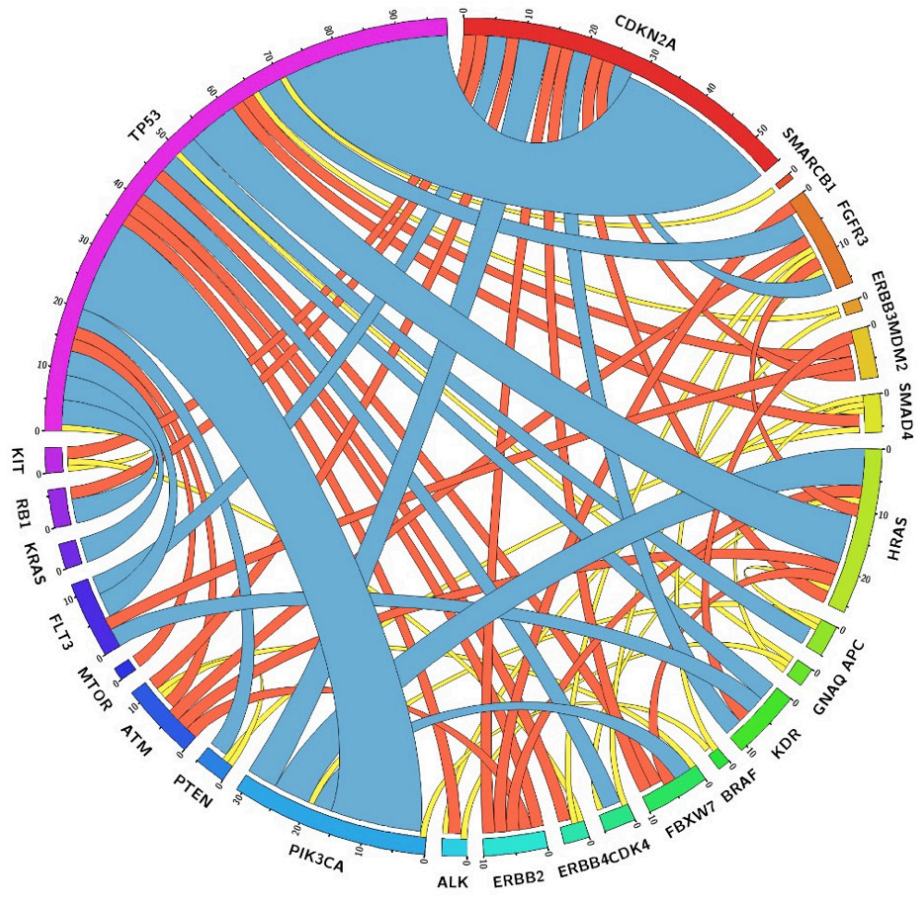

Figure 3. Circle plot illustrating co-mutations between genes found in HNSCC samples. Outer bars showing the absolute total amount of co-mutations found for genes entitling the bars. The curved ribbons inside the circle depict absolute number of co-mutations with the genes they are connected with (ribbon thickness corresponds with number of co-mutations). Ribbons are color coded by quartiles Q1 (yellow), Q2 (red), and Q3 (blue).

Mutational synonymy

Sequencing data of the associated (second) primary tumor were available for 51 of the 62 regional and distant metastases (the remaining 11 metastases were either associated to unsequenced primary tumors or were recurrent tumors only). By comparing the mutational profiles of the matched tumor pairs, we outlined the mutational heterogeneity of HNSCCs (Figure 4A). In 5 clinically related tumor pairs, no mutations were detected at all. The remaining 46 matched tumor pairs allowed for analysis of mutational synonymy, revealing 92% (81/88) of the analyzed gene mutations to be concordantly present in the associated metastasis. One discordant mutation was found in *PTEN*. A different single somatic nucleotide variant was detected in the metastasis (c.316G>T) compared to its associated primary tumor (c.892C>T). Six mutations were exclusively detected in the primary tumor: *HRAS* (c.38G>T), *TP53* (c.192_217del26), *PIK3CA* (c.3140A>G), *CDKN2A* (c.247C>G), *MDM2* (c.158G>A). Additionally, 2 new mutations were detected in exclusively the metastasis: *SMAD4* (c.725C>G) and *ALK* (c.1588G>C). *TP53* mutations were detected in 43 matched tumor pairs and were concordantly present in the associated metastasis in 95% (41/43). Mutational concordance of *CDKN2A, HRAS* and *PIK3CA* was respectively 92% (11/12), 83% (5/6) and 83% (5/6). An overview of mutational profiles of associated primary tumors and metastases is shown in supplementary table S4.

Twenty-five associated pairs of primary tumors and recurrences were available for comparison (Figure 4B). In 2 matched tumor pairs, no mutations were detected. In the other 23 matched tumor pairs, 89% (33/37) of gene mutations found in the primary tumors were also found in the associated recurrences. No discordant mutations were detected. Four mutations were exclusively detected in the primary tumor: *PTEN* (c.892C>T), *TP53* (c.192_217del26), *HRAS* (c.34G>A), *ERBB3* (c.1016G>A). Additionally, 5 new mutations were exclusively detected in the recurrent tumor sample: *FLT3* (c.2498C>G), *CDKN2A* (c.172C>T), *KIT* (c.1640A>G), *TP53* (c.406delC), *HRAS* (c.38G>T). *TP53* mutations were found concordant in 91% (21/23), 100% (4/4) in *PIK3CA*, 100% (2/2) in *CDKN2A*, and 50% (1/2) in *HRAS*. An overview of mutational profiles of associated primary tumors and recurrences is shown in supplementary table S5.

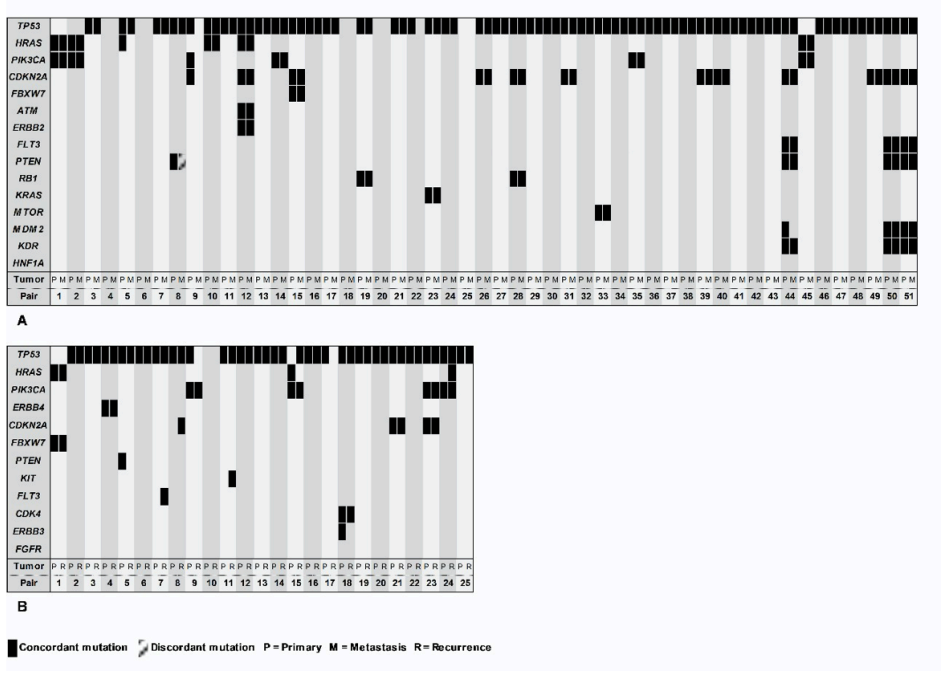

FIGURE 4. Block chart showing the mutational synonymy within tumor pairs. Mutational profiles of primary tumors (P) on the left side of vertical bars, compared to metastases (M) in (A), and recurrences (R) in (B) on the right side of the bars. Metastases and recurrences were either matched to a primary tumor or a second primary tumor.

DISCUSSION

Despite developments in diagnostic techniques and treatment, prognosis barely improved over the past few decades [8]. This is probably due to likelihood of recurrence (25-50%) after (chemo)radiotherapy and the lack of specific detection methods for recurrent disease [8, 13, 32]. And, although treatment options for recurrences depend on many different factors (e.g. initial disease stage, site of recurrence, previous treatment, expected quality of life), early detection of recurrences using biomarkers might contribute in improving disease outcome. Therefore, we think that the potential role of genetic biomarkers in the management of advanced stage HNSCC is of increasing importance. Although its potential role as a prognostic and predictive biomarker has been debated [33, 34], as well as its use for diagnosing recurrent or minimal residual disease during follow-up after treatment [35-38], *TP53* has, to our knowledge, not yet been proposed as a potential diagnostic biomarker for the early detection of locoregional metastases of HNSCC. Our data indicate that, using our 36-54 gene cancer panels, *TP53* is the most frequently mutated gene in HNSCC tumor samples. This was the case for primary tumors of all sites, as well as their associated recurrences and metastases. While previous studies of the mutational profile of HPV-negative HNSCC have shown prevalence rates of *TP53* mutations of 53%-78% [39, 40], these studies included either smaller sample sizes or were based on less sensitive whole exome sequencing (WES) data. More recent WES data from The Cancer Genome Atlas Network showed *TP53* mutations in 86% of 279 HPV-negative HNSCC samples, which is more consistent with our data [5]. Moreover, NGS data, based on clinical assays that were capable of deep coverage of cancer-relevant genes, found *TP53* mutations in 87% [4] and 81% [41] of HPV-negative HNSCCs.

In our study, *TP53* mutations were detected in only 64% of the primary OSCCs compared to 80% or more in other sites. Because HPV-positive OPSCCs and *TP53* mutations are known to be mutually exclusive in the majority of cases, in most studies similarly lowered *TP53* mutation rates are being found in OPSCCs [16, 42, 43]. In our study, this difference between OSCCs and other sites may also be due to the low number of HPV-positive OPSCC tumor samples. Another explanation may be the presence of epigenetic aberrations, copy number variations (CNV), or mutations in unknown genes that could have been more abundant in OSCCs than in tumors from other sites [44-46]. Also, differences in exon coverage of our gene panels could have affected the variability in mutation profiles of tumor samples, although the differences we found in *TP53* mutations cannot be fully proven because samples were not tested for all possible genetic aberrations and no statistical validation was performed. Additionally, statistical sampling bias could have affected sample sizes of HNSCC sites, since tumor type definition was primarily based on *TP53* clonality assessment. Moreover, because distinction between a recurrence and a second primary tumor is not always clear in clinical practice, the parameters of “time span” and “adjacent site”, used to determine whether a tumor is secondary or recurrent, may be interpreted differently in various studies. Re-evaluation of the available clinicopathological data did not yield any changes in *TP53* mutation prevalence rates.

Although not identified in hypopharyngeal tumors, we could confirm *CDKN2A* mutations as second most frequent mutations overall in HPV-negative HNSCCs. This is equivalent to findings in previous studies [4]. Compared to the literature, *HRAS* mutated less frequently in OSCC [5]. Furthermore, concordant with previous reports on *PIK3CA* [47], compared to other sites OSCCs most frequently contained *PIK3CA* mutations. Interestingly, concurrent PI3K pathway mutations such as *PIK3R1/PIK3R2* were recently identified as being involved in HNSCC tumor progression. This supports the potential use of mutations in this oncogenic pathway as predictive biomarkers [48]. As expected, fewer *TP53* mutations were found in HNSCCs of non-drinking and non-smoking patients. All tumor subgroups exclusively

related to alcohol and/or smoking contained the most *TP53* mutations. Furthermore, we found a relative increase of *PIK3CA* mutations in the non-smoking/non-drinking related group. NOTCH1 might also be a potential target as a potential diagnostic biomarker. *NOTCH1* mutations are found in 14-20% of HNSCC and possibly play a role as early drivers in OSCC progression [2-4, 49, 50]. However, we found no aberrations in the *NOTCH1* pathway. Aberrations were identified in the study of Agrawal *et al.*, in which tumor specimens were sequenced using WES based on assays that covered *NOTCH1* exons 1-34. This difference in results might be because our gene panels only covered *NOTCH1* exons 25, 27, and 37. Furthermore, it might be due to our gene panels not covering *NOTCH2* and *NOTCH3* at all. Clarifying tumor evolution genetically is of great importance, since tumor heterogeneity could seriously challenge the principle of using genetic mutations as (diagnostic) biomarkers [51, 52]. In order to use tumor specific mutations for quantifying purposes, it is essential to target mutations in ctDNA that are contained in both the primary tumor and its clonal expansions. Primary tumor biopsy (e.g. core needle, incision, or excision biopsy) carries the risk of incompletely depicting the mutational profile of primary tumor tissue due to intratumoral heterogeneity, a problem that increases with newly acquired mutations in clonal expansions. As a result, it can lead to tumor specific mutations being selected as biomarkers that are not present in ctDNA from clonal expansions. However, blood testing for these diagnostic biomarkers could identify mutations in *TP53* as well as those in other early driver genes that are extensively present in primary tumors and their clonal expansions.

Our data show that in most tumor pairs, mutations are concordant. This is largely consistent with the results of Hedberg et al. [53], who found that the primary tumor transmitted 86% of single somatic nucleotide variants identified in synchronous nodal metastases and 60% of those in recurrences. The relatively higher total amount of concordant mutations in associated recurrences compared to metastases in our study could be explained by the use of targeted gene panels instead of WES, which possibly impeded the detection of (unknown) driver genes that contribute to different pathways in tumor progression towards recurrences. Simultaneously, targeted sequencing could possibly have concealed intertumor heterogeneity, since we found comparable mutational synonymy rates of metastases and recurrences. On the other hand, our gene panels allowed for more sensitive sequencing compared to WES [54]. Thus, the small differences we found in mutational synonymy of *TP53* might suggest increased intertumor heterogeneity between primary tumors and recurrences compared to primary tumors and metastases. Another explanation for mutational discordance could be differences in tissue acquisition methods, because sometimes sequencing of primary tumor samples was performed on resection specimens, whereas sequencing of metastases was more often performed on (smaller) biopsies. This could have caused discordance due to intratumor heterogeneity.

Interestingly, the detection of two additional mutations (*SMAD4* and *ALK*) in two

metastatic samples, might implicate these mutations to drive metastatic outgrowth, as these mutations, especially *SMAD4*, contribute to the downregulation of growth inhibitors and increased genomic instability [55]. Though, the number of mutations we found is not definite to draw conclusions. Furthermore, technical difficulties and flaws associated with performing NGS on FFPE material might have biased our results, as fragmented DNA originating from FFPE tissue challenges sequencing. Therefore, used NGS assays are adapted by using small amplicons facilitating shorter fragment sequencing. Also, fixation of tissue is known to potentially deaminate cytosines, possibly leading to more C>T or G>A base transitions [56]. However, recently performed validation of our gene panels revealed minimal FFPE induced DNA damage [57].

Despite their limitations, our findings provide useful information for developing new diagnostic strategies for HNSCC using targeted NGS panels that are easily accessible and capable of deep sequencing. Most investigated gene mutations were found concordantly mutated in the associated metastases and recurrences. Furthermore, *TP53* mutations are by far the most frequent. This suggests *TP53* mutations have potential value as diagnostic biomarkers in conjunction with subsequent ctDNA detection through liquid biopsy. By depicting these mutations in ctDNA using liquid biopsies, tumor remission after treatment could possibly be monitored non-invasively as compared to repeated biopsy for histological confirmation. This might complement current surveillance methods of clinical evaluation supported by flexible endoscopy and/or imaging such as PET-CT or diffusion weighted MRI, in order to increase accuracy for early detection of recurrent and/or metastatic HNSCC in the future.

MATERIAL AND METHODS

Data collection and analysis

We collected NGS sequencing data of all HNSCC samples, generated through TP53 clonality assessment on clinical request between the period of October 2013 and May 2015. Sequencing results from primary skin tumors of the head and neck region were not included. All samples on which sequencing was performed were formalin-fixed paraffin embedded (FFPE), after being obtained by surgical resection or tissue biopsy for diagnostic purposes between March 1992 to April 2015. Demographic and clinical data, including history of tobacco and alcohol use, were retrieved from hospital charts. Smoking and alcohol consumption habits were classified as previously described [58].

For analysis, samples were grouped and sorted by site of primary tumor (i.e. oral cavity, oropharynx, hypopharynx, larynx, miscellaneous), and tumor subtype (primary, metastasis,

recurrence). Definition of tumor type (i.e. primary tumor, metastasis or recurrence) was mainly based on *TP53* clonality. If a clonal relationship could not be ruled out, we based subtype determination on clinical suspicion and date of incidence as described previously [58]. In the same manner, distinction was made between second primary tumors, metastases and recurrences. Samples were excluded from analysis if tumor subtype remained unclear. Also, samples of unknown anatomical origin and duplicates of sequencing results were excluded.

Sequencing results were retrieved from the nationwide network and registry of histo- and cytopathology in The Netherlands (PALGA). Descriptive analysis consisted of mutational prevalence, which was determined for each gene in primary tumors and for all tumor types. Because of the use of varying gene panels over time by our molecular diagnostics laboratory, all (average) percentages were weighed for differences in gene coverage of used gene panels. Furthermore, co-occurrence of gene mutations in primary tumors was determined. Mutational synonymy was assessed by comparing the genetic profiles of primary tumors or second primary tumors with matched locoregional and/or distant metastases, if present. Gene mutations within matched tumor pairs were considered concordant when alterations were identical in the primary tumor and its associated metastasis or recurrence. Associated tumor samples within each matched pair were consistently sequenced by the same gene panel.

Molecular Analysis

Clonality assessment was based on the presence of *TP53* mutations or similar loss of heterozygosity (LOH) profiles using short tandem repeats. Targeted NGS was performed using the Ion Torrent™ PGM platform (Thermo Fisher Scientific, Waltham, MA, USA) as previously described [57]. The following gene panels were used: CHPv2 (Thermo Fisher Scientific, Waltham, MA, USA), CHPv2+ (i.e. CHPv2 supplemented with several extra genes and amplicons) and OAPv2 [59]. Exact genes and exons sequenced are shown in supplementary table S6-8. References used for reporting gene mutations were Center for Personalized Cancer Treatment (CPCT, Utrecht, The Netherlands; http://www.cpct.nl), Catalogue Of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic), International Cancer Genome Consortium (ICGC; https://icgc.org/) and The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov/). Sufficient coverage is reached when an amplicon was sequenced at least 500 times. Variants with an allele frequency below 1% were considered as background noise and were not reported. Variants with allele frequency between 1% and 5% were first discussed multidisciplinary before decision to report. Variants with allele frequencies above 5% were reported. The used assay was validated recently according to general rules for diagnostic laboratories through ISO certification [57]. Accordingly, minimum tumor percentage is set at 10%. Gene amplification was indicated when five or more amplicons showed a z-score of 5 or more [59]. Where NGS failed, additional Sanger sequencing of *TP53* exon 4-9 in forward and reverse directions was performed to allow for *TP53* clonality assessment. The Sanger sequencing products were analyzed on a 3730 DNA Analyzer (Applied Biosystems, Foster city, CA, USA). If sequencing failed altogether, samples were ultimately excluded from analysis.

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Conflict of Interest statement

The authors declare no conflicts of interest.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer Journal international du cancer. 2015;136:E359-86.
2. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333:1154-7.
3. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333:1157-60.
4. Chung CH, Guthrie VB, Masica DL, Tokheim C, Kang H, Richmon J, et al. Genomic alterations in head and neck squamous cell carcinoma determined by cancer gene- targeted sequencing. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26:1216-23.
5. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517:576-82.
6. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. The Lancet Oncology. 2010;11:781-9.
7. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. The New England journal of medicine. 2010;363:24-35.
8. Braakhuis BJ, Leemans CR, Visser O. Incidence and survival trends of head and neck squamous cell carcinoma in the Netherlands between 1989 and 2011. Oral oncology. 2014;50:670-5.
9. Chung CH, Parker JS, Ely K, Carter J, Yi YJ, Murphy BA, et al. Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor- kappa B signaling as characteristics of a high-risk head and neck squamous cell carcinoma. Cancer research. 2006;66:8210-8.
10. Chung CH, Parker JS, Karaca G, Wu JY, Funkhouser WK, Moore' D, et al. Molecular classification of head and neck squamous cell carcinomas using patterns of gene expression. Cancer Cell. 2004;5:489-500.
11. Graveland AP, Braakhuis BJ, Eerenstein SE, de Bree R, Bloemena E, de Maaker M, et al. Molecular diagnosis of minimal residual disease in head and neck cancer patients. Cell Oncol (Dordr). 2012;35:367-75.
12. Smeets SJ, Brakenhoff RH, Ylstra B, van Wieringen WN, van de Wiel MA, Leemans CR, et al. Genetic classification of oral and oropharyngeal carcinomas identifies subgroups with a different prognosis. Cellular oncology : the official journal of the International Society for Cellular Oncology. 2009;31:291-300.
13. Ho AS, Kraus DH, Ganly I, Lee NY, Shah JP, Morris LG. Decision making in the management of recurrent head and neck cancer. Head & neck. 2014;36:144-51.
14. Leemans CR, Tiwari R, Nauta JJP, Vanderwaal I, Snow GB. Recurrence at the Primary Site in Head and Neck-Cancer and the Significance of Neck Lymph-Node Metastases as a Prognostic Factor. Cancer. 1994;73:187-90.
15. Graveland AP, Golusinski PJ, Buijze M, Douma R, Sons N, Kuik DJ, et al. Loss of heterozygosity at 9p and p53 immunopositivity in surgical margins predict local relapse in head and neck squamous cell carcinoma. International Journal of Cancer. 2011;128:1852-9.
16. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nature reviews Cancer. 2011;11:9-22.
17. helling A, Muthukaruppan A, Lasham A, Blenkiron C, Laking G, et al. Predictive and prognostic molecular markers for cancer medicine. Therapeutic advances in medical oncology. 2010;2:125-48.
18. Noorlag R, van Kempen PM, Stegeman I, Koole R, van Es RJ, Willems SM. The diagnostic value of 11q13 amplification and protein expression in the detection of nodal metastasis from oral squamous cell carcinoma: a systematic review and meta-analysis. Virchows Archiv : an international journal of pathology. 2015;466:363-73.
19. Kang H, Kiess A, Chung CH. Emerging biomarkers in head and neck cancer in the era of genomics. Nature reviews Clinical oncology. 2015;12:11-26.
20. Thariat J, Vignot S, Lapierre A, Falk AT, Guigay J, Van Obberghen-Schilling E, et al. Integrating genomics in head and neck cancer treatment: Promises and pitfalls. Crit Rev Oncol Hematol. 2015;95:397-406.
21. Nees M, Homann N, Discher H, Andl T, Enders C, Herold-Mende C, et al. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. Cancer research. 1993;53:4189-96.
22. Califano J, vanderRiet P, Westra W, Nawroz H, Clayman G, Piantadosi S, et al. Genetic progression model for head and neck cancer: Implications for field cancerization. Cancer research. 1996;56:2488-92.
23. Gollin SM. Chromosomal alterations in squamous cell carcinomas of the head and neck: window to the biology of disease. Head & neck. 2001;23:238-53.
24. Braakhuis BJ, Leemans CR, Brakenhoff RH. A genetic progression model of oral cancer: current evidence and clinical implications. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2004;33:317-22.
25. Boyle JO, Hakim J, Koch W, van der Riet P, Hruban RH, Roa RA, et al. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer research. 1993;53:4477-80.
26. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer. 1953;6:963-8.
27. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer research. 2003;63:1727-30.
28. Thierry AR, Mouliere F, Gongora C, Ollier J, Robert B, Ychou M, et al. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. Nucleic Acids Res. 2010;38:6159-75.
29. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nature Medicine. 2008;14:985-90.
30. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget. 2015;6:42008-18.
31. Heidary M, Auer M, Ulz P, Heitzer E, Petru E, Gasch C, et al. The dynamic range of circulating tumor DNA in metastatic breast cancer. Breast Cancer Research. 2014;16.
32. de Bree R, van der Putten L, Brouwer J, Castelijns JA, Hoekstra OS, Leemans CR. Detection of locoregional recurrent head and neck cancer after (chemo)radiotherapy using modern imaging. Oral oncology. 2009;45:386-93.
33. Nemunaitis J, Clayman G, Agarwala SS, Hrushesky W, Wells JR, Moore C, et al. Biomarkers Predict p53 Gene

Therapy Efficacy in Recurrent Squamous Cell Carcinoma of the Head and Neck. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15:7719-25.

34. Masica DL, Li S, Douville C, Manola J, Ferris RL, Burtness B, et al. Predicting survival in head and neck squamous cell carcinoma from TP53 mutation. Human genetics. 2015;134:497-507.
35. van Houten VMM, Tabor MP, van den Brekel MWM, Kummer JA, Denkers F, Dijkstra J, et al. Mutated p53 as a molecular marker for the diagnosis of head and neck cancer. Journal of Pathology. 2002;198:476-86.
36. Wang X, Chen S, Chen X, Zhang C, Liang X. Tumor-related markers in histologically normal margins correlate with locally recurrent oral squamous cell carcinoma: a retrospective study. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2016;45:83-8.
37. Poeta ML, Manola J, Goldenberg D, Forastiere A, Califano JA, Ridge JA, et al. The Ligamp TP53 Assay for Detection of Minimal Residual Disease in Head and Neck Squamous Cell Carcinoma Surgical Margins. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15:7658-65.
38. Lopez M, Aguirre JM, Cuevas N, Anzola M, Videgain J, Aguirregaviria J, et al. Use of cytological specimens for p53 gene alteration detection in oral squamous cell carcinoma risk patients. Clin Oncol-Uk. 2004;16:366-70.
39. Mountzios G, Rampias T, Psyrri A. The mutational spectrum of squamous-cell carcinoma of the head and neck: targetable genetic events and clinical impact. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2014;25:1889-900.
40. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. The New England journal of medicine. 2007;357:2552-61.
41. Seiwert TY, Zuo ZX, Keck MK, Khattri A, Pedamallu CS, Stricker T, et al. Integrative and Comparative Genomic Analysis of HPV-Positive and HPV-Negative Head and Neck Squamous Cell Carcinomas. Clinical Cancer Research. 2015;21:632-41.
42. Pickering CR, Zhang JX, Yoo SY, Bengtsson L, Moorthy S, Neskey DM, et al. Integrative Genomic Characterization of Oral Squamous Cell Carcinoma Identifies Frequent Somatic Drivers. Cancer discovery. 2013;3:770-81.
43. Mutational landscape of gingivo-buccal oral squamous cell carcinoma reveals new recurrently-mutated genes and molecular subgroups. Nature communications. 2013;4:2873.
44. Noorlag R, van Kempen PMW, Moelans CB, de Jong R, Blok LER, Koole R, et al. Promoter hypermethylation using 24-gene array in early head and neck cancer Better outcome in oral than in oropharyngeal cancer. Epigenetics. 2014;9:1220-7.
45. van Kempen PM, Noorlag R, Braunius WW, Moelans CB, Rifi W, Savola S, et al. Clinical relevance of copy number profiling in oral and oropharyngeal squamous cell carcinoma. Cancer medicine. 2015;4:1525-35.
46. Guerrero-Preston R, Michailidi C, Marchionni L, Pickering CR, Frederick MJ, Myers JN, et al. Key tumor suppressor genes inactivated by “greater promoter” methylation and somatic mutations in head and neck cancer. Epigenetics. 2014;9:1031-46.
47. Murugan AK, Hong NT, Fukui Y, Munirajan AK, Tsuchida N. Oncogenic mutations of the PIK3CA gene in head and neck squamous cell carcinomas. International journal of oncology. 2008;32:101-11.
48. Lui VW, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. Cancer discovery. 2013;3:761-9.
49. William WN, Jr., El-Naggar AK. A novel target for oral cancer chemoprevention? Notch quite, yet. Cancer Prev Res (Phila). 2015;8:262-5.
50. Izumchenko E, Sun K, Jones S, Brait M, Agrawal N, Koch W, et al. Notch1 mutations are drivers of oral tumorigenesis. Cancer Prev Res (Phila). 2015;8:277-86.
51. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. The New England journal of medicine. 2012;366:883-92.
52. Jacoby MA, Duncavage EJ, Walter MJ. Implications of Tumor Clonal Heterogeneity in the Era of Next-Generation Sequencing. Trends in Cancer. 2016;1:10.
53. Hedberg ML, Goh G, Chiosea SI, Bauman JE, Freilino ML, Zeng Y, et al. Genetic landscape of metastatic and recurrent head and neck squamous cell carcinoma. The Journal of clinical investigation. 2016;126:169-80.
54. Gagan J, Van Allen EM. Next-generation sequencing to guide cancer therapy. Genome medicine. 2015;7:80.
55. Korc M. Smad4: gatekeeper gene in head and neck squamous cell carcinoma. The Journal of clinical investigation. 2009;119:3208-11.
56. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. The American journal of pathology. 2002;161:1961- 71.
57. de Leng WW, Gadellaa-van Hooijdonk CG, Barendregt-Smouter FA, Koudijs MJ, Nijman I, Hinrichs JW, et al. Targeted Next Generation Sequencing as a Reliable Diagnostic Assay for the Detection of Somatic Mutations in Tumours Using Minimal DNA Amounts from Formalin Fixed Paraffin Embedded Material. PloS one. 2016;11:e0149405.
58. van Kempen PM, Verdam FJ, de Poel E, Braunius WW, de Weger RA, van Es RJ, et al. TP53 Y220C Is a Hotspot Mutation in Oropharyngeal Squamous Cell Carcinoma. Pathobiology : journal of immunopathology, molecular and cellular biology. 2015;82:21- 7.
59. Hoogstraat M, Hinrichs JW, Besselink NJ, Radersma-van Loon JH, de Voijs CM, Peeters T, et al. Simultaneous detection of clinically relevant mutations and amplifications for routine cancer pathology. The Journal of molecular diagnostics : JMD. 2015;17:10-8.

SUPPLEMENTARY DATA

TABLE S1. Gene mutation count in primary HNSCC samples per site

	OPSCC (%)		OSCC (%)		HSCC (%)		LSCC (%)		Misc (%)	
TP53	30	(81)	34	(64)	13	(81)	24	(86)	12	(80)
CDKN2A	1	(3)	7	(15)	0	(0)	3	(12)	3	(21)
PIK3CA	1	(3)	10	(22)	1	(7)	1	(4)	1	(7)
HRAS	3	(10)	5	(11)	1	(7)	0	(0)	1	(7)
FBXW7	1	(4)	1	(3)	0	(0)	1	(5)	0	(0)
FGFR3	1	(4)	1	(3)	0	(0)	0	(0)	1	(8)
SMAD4	0	(0)	0	(0)	0	(0)	1	(5)	1	(6)
ERBB4	0	(0)	0	(0)	0	(0)	0	(0)	1	(7)
BRAF	0	(0)	1	(2)	0	(0)	0	(0)	1	(6)
APC	0	(0)	0	(0)	0	(0)	0	(0)	1	(6)
ALK	0	(0)	0	(0)	0	(0)	1	(4)	1	(6)
GNAQ	0	(0)	0	(0)	0	(0)	1	(4)	1	(6)
PTEN	1	(3)	1	(2)	0	(0)	0	(0)	1	(6)
ATM	1	(4)	0	(0)	0	(0)	0	(0)	1	(8)
ERBB2	0	(0)	0	(0)	0	(0)	0	(0)	2	(13)
KIT	0	(0)	0	(0)	0	(0)	0	(0)	1	(6)
FLT3	0	(0)	1	(3)	0	(0)	0	(0)	0	(0)
RB1	0	(0)	1	(3)	1	(9)	0	(0)	1	(6)
KRAS	1	(3)	0	(0)	0	(0)	0	(0)	1	(6)
MTOR	0	(0)	1	(13)	0	(0)	0	(0)	1	(6)
SMARCB1	0	(0)	0	(0)	0	(0)	1	(5)	1	(6)
CDK4	1	(25)	0	(0)	1	(25)	0	(0)	1	(6)
ERBB3	0	(0)	0	(0)	1	(25)	0	(0)	1	(6)
MDM2	0	(0)	1	(7)	0	(0)	0	(0)	1	(6)
KDR	0	(0)	1	(2)	0	(0)	0	(0)	1	(6)
HNF1A	0	(0)	0	(0)	0	(0)	0	(0)	1	(8)

TABLE S2. Gene mutation count in HNSCC samples per type

	Primary (%)		Recurrence (%)		Metastasis (%)	
TP53	113	(76)	50	(82)	24	(83)
CDKN2A	12	(9)	11	(21)	4	(14)
PIK3CA	14	(11)	5	(10)	5	(18)
HRAS	9	(7)	3	(6)	2	(7)
FBXW7	4	(4)	1	(2)	1	(5)
FGFR3	2	(2)	1	(2)	0	(0)
BRAF	0	(0)	1	(2)	1	(4)
APC	0	(0)	1	(2)	2	(7)
PTEN	2	(2)	1	(2)	1	(4)
CDK4	1	(5)	0	(0)	1	(14)
ERBB4	0	(0)	0	(0)	1	(4)
ATM	2	(2)	1	(2)	0	(0)
ERBB2	1	(1)	1	(2)	0	(0)
SMAD4	0	(0)	1	(2)	0	(0)
FLT3	1	(1)	2	(4)	1	(4)
ALK	1	(1)	1	(2)	0	(0)
KRAS	1	(1)	3	(4)	0	(0)
MTOR	0	(0)	1	(9)	0	(0)
SMARCB1	0	(0)	0	(0)	0	(0)
GNAQ	1	(1)	0	(0)	0	(0)
ERBB3	1	(5)	0	(0)	0	(0)
MDM2	0	(0)	1	(9)	0	(0)
KDR	1	(1)	2	(4)	0	(0)
HNF1A	1	(1)	0	(0)	0	(0)
RB1	1	(1)	2	(5)	0	(0)
KIT	0	(0)	0	(0)	1	(4)

TABLE S3. Absolute numbers of co-mutations between genes

Genes	HNF1A	KDR	MDM2	ERBB3	CDK4	MTOR	KRAS	FLT3	KIT	ERBB2	FGFR3	BRAF	ERBB4	PIK3CA	HRAS	GNAQ	ALK	SMAD4	SMARCB1	RB1	ATM	PTEN	FBXW7	CDKN2A	APC
TP53	-	3	2	1	3	2	4	4	1	2	4	-	2	15	8	1	2	2	1	4	2	3	2	27	3
APC	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-
CDKN2A	-	3	2	-	-	-	-	3	2	2	3	-	-	5	2	-	-	-	-	2	2	-	2	-	-
FBXW7	-	-	-	-	-	-	-	-	-	-	-	-	-	3	2	-	-	-	-	-	1	-	-	-	-
PTEN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-
ATM	-	-	-	-	-	-	-	-	-	2	2	-	-	-	2	-	-	-	-	-	-	-	-	-	-
RB1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SMARCB1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SMAD4	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	1	-	-	-	-	-	-	-	-
ALK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
GNAQ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HRAS	-	-	-	-	1	-	-	-	-	2	2	-	-	6	-	-	-	-	-	-	-	-	-	-	-
PIK3CA	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
ERBB4	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BRAF	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FGFR3	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERBB2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KIT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FLT3	-	3	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KRAS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MTOR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDK4	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERBB3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MDM2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KDR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE S4. Tumor pairs

PRIMARY TUMOR				METASTASIS			
Pair	Gene	Mutation	Type	Pair	Gene	Mutation	Type
1	HRAS	c.181C>A	MISSENSE	1	HRAS	c.181C>A	MISSENSE
1	PIK3CA	c.1633G>A	MISSENSE	1	PIK3CA	c.1633G>A	MISSENSE
2	HRAS	c.181C>A	MISSENSE	2	HRAS	c.181C>A	MISSENSE
2	PIK3CA	c.1633G>A	MISSENSE	2	PIK3CA	c.1633G>A	MISSENSE
3	TP53	c.536A>G	MISSENSE	3	TP53	c.536A>G	MISSENSE
4	N/A	N/A	N/A	4	N/A	N/A	N/A
5	TP53	c.838A>G	MISSENSE	5	TP53	c.838A>G	MISSENSE
5	HRAS	c.38G>T	MISSENSE	6	N/A	N/A	N/A
6	N/A	N/A	N/A	7	TP53	c.306C>A	MISSENSE
7	TP53	c.306C>A	MISSENSE	7	TP53	c.310C>T	TRUNC
7	TP53	c.310C>T	TRUNC	8	TP53	c.490A>G	MISSENSE
8	TP53	c.490A>G	MISSENSE	8	TP53	c.489C>A	TRUNC
8	TP53	c.489C>A	TRUNC	8	SMAD4	c.725C>G	TRUNC
8	PTEN	c.892C>T	TRUNC	8	PTEN	c.316G>T	TRUNC
9	TP53	c.659A>G	MISSENSE	9	N/A	N/A	N/A
9	PIK3CA	c.3140A>G	MISSENSE	10	TP53	c.733G>A	MISSENSE
9	CDKN2A	c.247C>G	MISSENSE	10	HRAS	c.172C>T	TRUNC
10	TP53	c.733G>A	MISSENSE	11	TP53	c.473G>T	MISSENSE
10	HRAS	c.172C>T	TRUNC	12	TP53	c.799C>G	MISSENSE
11	TP53	c.473G>T	MISSENSE	12	HRAS	c.183G>T	MISSENSE
11	TP53	c.192_217del26	INFRAME	12	CDKN2A	c.238C>T	TRUNC
12	TP53	c.799C>G	MISSENSE	12	FGFR3	c.1108G>A	MISSENSE
12	HRAS	c.183G>T	MISSENSE	12	ATM	c.9124C>T	MISSENSE
12	CDKN2A	c.238C>T	TRUNC	12	ERBB2	c.2593- 2594GG>AA	MISSENSE
12	FGFR3	c.1108G>A	MISSENSE	13	TP53	c.818G>T	MISSENSE
12	ATM	c.9124C>T	MISSENSE	14	TP53	c.659A>G	MISSENSE
12	ERBB2	c.2593- 2594GG>AA	MISSENSE	14	PIK3CA	c.1624G>A	MISSENSE
13	TP53	c.818G>T	MISSENSE	15	TP53	c.730G>T	MISSENSE
14	TP53	c.659A>G	MISSENSE	15	CDKN2A	c.192_194delGC T	MISSENSE
14	PIK3CA	c.1624G>A	MISSENSE	15	FBXW7	c.1273C>G	MISSENSE
15	TP53	c.730G>T	MISSENSE	16	TP53	c.583delA	INFRAME
15	CDKN2A	c.192_194delGCT	MISSENSE	17	TP53	c.920-1G>T	UNKNOWN
15	FBXW7	c.1273C>G	MISSENSE	18	N/A	N/A	N/A
16	TP53	c.583delA	INFRAME	19	TP53	c.841_842del2	INFRAME
17	TP53	c.920-1G>T	UNKNOWN	19	RB1	c.2107-1G>C	UNKNOWN
18	N/A	N/A	N/A	20	N/A	N/A	N/A
19	TP53	c.841_842del2	INFRAME	21	TP53	c.743G>A	MISSENSE

TABLE S4 CONTINUED.

	PRIMARY TUMOR				METASTASIS		
Pair	**Gene**	**Mutation**	**Type**	**Pair**	**Gene**	**Mutation**	**Type**
19	RB1	c.2107-1G>C	UNKNOWN	22	N/A	N/A	N/A
20	N/A	N/A	N/A	23	TP53	c.105del5	INFRAME
21	TP53	c.743G>A	MISSENSE	23	KRAS	c.38G>A	MISSENSE
22	TP53	c.379T>C	MISSENSE	24	TP53	c.396G>T	MISSENSE
23	TP53	c.105del5	INFRAME	25	N/A	N/A	N/A
23	KRAS	c.38G>A	MISSENSE	26	TP53	c.551delA	INFRAME
24	TP53	c.396G>T	MISSENSE	26	CDKN2A	c.205G>T	TRUNC
25	N/A	N/A	N/A	27	TP53	c.592G>T	TRUNC
26	TP53	c.551delA	INFRAME	28	TP53	c.524G>A	MISSENSE
26	CDKN2A	c.205G>T	TRUNC	28	CDKN2A	c.172C>T	TRUNC
7	TP53	c.592G>T	TRUNC	28	RB1	c.1698+6T>G	UNKNOWN
28	P53	c.524G>A	MISSENSE	29	TP53	c.225-35G>C	UNKNOWN
28	CDKN2A	c.172C>T	TRUNC	30	TP53	c.225-35G>C	UNKNOWN
28	RB1	c.1698+6T>G	UNKNOWN	31	TP53	c.742C>T	MISSENSE
29	TP53	c.225-35G>C	UNKNOWN	31	TP53	c.764_765delTCi nsAT	MISSENSE
30	TP53	c.225-35G>C	UNKNOWN	31	CDKN2A	c.172C>T	TRUNC
31	TP53	c.742C>T	MISSENSE	32	TP53	c.329G>T	MISSENSE
31	TP53	c.764_765delTCin sAT	MISSENSE	32	TP53	c.637C>T	TRUNC
32	TP53	c.329G>T	MISSENSE	33	TP53	c.505_506insT	INFRAME
32	TP53	c.637C>T	TRUNC	33	TP53	c.769C>G	MISSENSE
33	TP53	c.505_506insT	INFRAME	33	MTOR	c.34G>A	MISSENSE
33	TP53	c.769C>G	MISSENSE	34	TP53	c.101dupC	INFRAME
33	MTOR	c.34G>A	MISSENSE	35	TP53	c.818G>A	MISSENSE
34	TP53	c.101dupC	INFRAME	35	PIK3CA	c.1624G>A	MISSENSE
35	TP53	c.818G>A	MISSENSE	36	TP53	c.700T>C	MISSENSE
35	PIK3CA	c.1624G>A	MISSENSE	36	ALK	c.1588G>C	MISSENSE
36	TP53	c.700T>C	MISSENSE	37	TP53	c.700T>C	MISSENSE
37	TP53	c.700T>C	MISSENSE	38	TP53	c.637C>T	TRUNC
38	TP53	c.637C>T	TRUNC	39	TP53	c.733G>A	MISSENSE
39	TP53	c.733G>A	MISSENSE	39	CDKN2A	c.172C>T	TRUNC
40	TP53	c.733G>A	MISSENSE	40	TP53	c.733G>A	MISSENSE
40	CDKN2A	c.172C>T	TRUNC	40	CDKN2A	c.172C>T	TRUNC
41	TP53	c.476delC	INFRAME	41	TP53	c.476delC	INFRAME
42	TP53	c.743G>C	MISSENSE	42	TP53	c.743G>C	MISSENSE
43	TP53	c.457_460del4	INFRAME	43	TP53	c.457_460del4	INFRAME
43	TP53	c.375_375+1del2i ns2	UNKNOWN	43	TP53	c.375_375+1del 2ins2	UNKNOWN
44	TP53	c.734G>A	INFRAME	44	TP53	c.734G>A	INFRAME

TABLE S4 CONTINUED.

	PRIMARY TUMOR				METASTASIS		
Pair	**Gene**	**Mutation**	**Type**	**Pair**	**Gene**	**Mutation**	**Type**
44	CDKN2A	c.151-1G>A	UNKNOWN	44	CDKN2A	c.151-1G>A	UNKNOWN
44	FLT3	c.505A>G	MISSENSE	44	FLT3	c.505A>G	MISSENSE
44	MDM2	c.158G>A	MISSENSE	44	KDR	c.2630G>A	MISSENSE
44	KDR	c.2630G>A	MISSENSE	45	HRAS	c.181C>A	MISSENSE
45	HRAS	c.181C>A	MISSENSE	45	PIK3CA	c.1633G>A	MISSENSE
45	PIK3CA	c.1633G>A	MISSENSE	46	TP53	c.743G>A	MISSENSE
46	TP53	c.743G>A	MISSENSE	47	TP53	c.396G>T	MISSENSE
47	TP53	c.396G>T	MISSENSE	48	TP53	c.700T>C	MISSENSE
48	TP53	c.700T>C	MISSENSE	49	TP53	c.733G>A	MISSENSE
49	TP53	c.733G>A	MISSENSE	49	CDKN2A	c.172C>T	TRUNC
49	CDKN2A	c.172C>T	TRUNC	50	TP53	c.734G>A	MISSENSE
50	CDKN2A	c.151-1G>A	UNKNOWN	50	CDKN2A	c.151-1G>A	UNKNOWN
50	FLT3	c.505A>G	MISSENSE	50	FLT3	c.505A>G	MISSENSE
50	MDM2	c.158G>A	MISSENSE	50	MDM2	c.158G>A	MISSENSE
50	KDR	c.2630G>A	MISSENSE	50	KDR	c.2630G>A	MISSENSE
51	TP53	c.734G>A	MISSENSE	51	TP53	c.734G>A	MISSENSE
51	CDKN2A	c.151-1G>A	UNKNOWN	51	CDKN2A	c.151-1G>A	UNKNOWN
51	FLT3	c.505A>G	MISSENSE	51	FLT3	c.505A>G	MISSENSE
51	MDM2	c.158G>A	MISSENSE	51	MDM2	c.158G>A	MISSENSE
51	KDR	c.2630G>A	MISSENSE	51	KDR	c.2630G>A	MISSENSE

TABLE S5. Tumor pairs

PRIMARY				RECURRENCE			
Patient	**Gene**	**Mutation**	**Type**	**Patient**	**Gene**	**Mutation**	**Type**
1	HRAS	c.38G>A	MISSENSE	1	HRAS	c.38G>A	MISSENSE
1	FBX7	c.1153C>T	MISSENSE	1	FBX7	c.1153C>T	MISSENSE
2	TP53	c.586C>T	TRUNC	2	TP53	c.586C>T	TRUNC
3	TP53	c.438G>A	TRUNC	3	TP53	c.438G>A	TRUNC
4	TP53	c.574C>T	TRUNC	4	TP53	c.574C>T	TRUNC
4	ERBB4	c.513C>A	MISSENSE	4	ERBB4	c.513C>A	MISSENSE
5	TP53	c.490A>G	MISSENSE	5	TP53	c.490A>G	MISSENSE
5	TP53	c.489C>A	TRUNC	5	TP53	c.489C>A	TRUNC
5	PTEN	c.892C>T	TRUNC	5	PTEN	c.892C>T	TRUNC
6	TP53	c.473G>T	MISSENSE	6	TP53	c.473G>T	MISSENSE
6	TP53	c.192_217del2 6	INFRAME	7	TP53	c.809T>G	MISSENSE
7	TP53	c.809T>G	MISSENSE	8	TP53	c.560-2A>G	UNKNOWN
8	TP53	c.560-2A>G	UNKNOWN	8	CDKN2A	c.172C>T	TRUNC
9	PIK3CA	c.3140A>G	MISSENSE	9	PIK3CA	c.3140A>G	MISSENSE
10	N/A	N/A	N/A	10	N/A	N/A	N/A
11	TP53	c.742C>T	MISSENSE	11	TP53	c.742C>T	MISSENSE
12	TP53	c.413C>T	MISSENSE	11	KIT	c.1640A>G	MISSENSE
13	TP53	c.527G>A	MISSENSE	12	TP53	c.413C>T	MISSENSE
14	TP53	c.734G>A	MISSENSE	13	TP53	c.614A>G	MISSENSE
15	HRAS	c.34G>A	MISSENSE	14	TP53	c.734G>A	MISSENSE
15	PIK3CA	c.3140A>G	MISSENSE	15	HRAS	c.34G>A	MISSENSE
16	TP53	c.707A>G	MISSENSE	15	PIK3CA	c.3140A>G	MISSENSE
16	TP53	c.560del1	INFRAME	16	TP53	c.707A>G	MISSENSE
17	N/A	N/A	N/A	16	TP53	c.560del1	INFRAME
18	TP53	c.584T>C	MISSENSE	17	N/A	N/A	N/A
18	CDK4	c.693del1	INFRAME	18	TP53	c.584T>C	MISSENSE
18	ERBB3	c.1016G>A	MISSENSE	18	CDK4	c.693del1	INFRAME
19	TP53	c.475G>C	MISSENSE	19	TP53	c.475G>C	MISSENSE
20	TP53	c.916C>T	TRUNC	20	TP53	c.916C>T	TRUNC
21	TP53	c.610G>T	TRUNC	21	TP53	c.610G>T	TRUNC
21	CDKN2A	c.238C>T	TRUNC	21	CDKN2A	c.238C>T	TRUNC
22	TP53	c.637C>T	TRUNC	22	TP53	c.637C>T	TRUNC
23	TP53	c.706_708del3	INFRAME	23	TP53	c.706_708del3	INFRAME
23	PIK3CA	c.1353del14ins 1	INFRAME	23	PIK3CA	c.1353del14ins 1	INFRAME
23	CDKN2A	c.172C>T	TRUNC	23	CDKN2A	c.172C>T	TRUNC
24	TP53	c.181G>A	MISSENSE	24	TP53	c.181G>A	MISSENSE
24	PIK3CA	c.1633G>A	MISSENSE	24	HRAS	c.38G>T	MISSENSE

TABLE S5 CONTINUED.

PRIMARY				RECURRENCE			
Patient	**Gene**	**Mutation**	**Type**	**Patient**	**Gene**	**Mutation**	**Type**
25	TP53	c.413C>T	MISSENSE	24	PIK3CA	c.1633G>A	MISSENSE
				25	TP53	c.413C>T	MISSENSE

TABLE S6. Gene coverage of Ion AmpliSeq™ OncoAmp Panel v2:

AKT1	exon 1,3,6,9,13	ESR1	exon 3-7,10	MET	exon 2,5,14,16,19
ALK	exon 2,5,8,12,23,25	FGFR1	exon 2,4,7,9,14,18	MTOR	exon 2,12,24,46,58
BRAF	exon 3,7,11,15,18	FGFR2	exon 2,4,7,9,14,18	MYC	exon 1-3
CDH1*	exon 1-16	FGFR4	exon 5,11,16,18	MYCN	exon 2,3
CDK4	exon 1-8	FLT3	exon 5,11,14,16,20	NRAS	exon 2,3
CDK6	exon 1-6,8	GNAS	exon 8	PDGFRA	exon 6,12,14,16,18,2,3
CDKN2A	exon 2	GNAQ	exon 5	PIK3CA	exon 1,4,7,9,13,20
CTNNB1	exon 3	HRAS	exon 2,3,5	PTEN*	exon 1-9
EGFR*	exon 1-28	KDR	exon 6,7,11,19,21,26,27,30	RET	exon 10,11,13,15,17,18
ERBB2	exon 5,11,22-24,30	KIT	exon 2,10,11,13-15,17,18	TOP2A	exon 2,8,14,22,28,34
ERBB3	exon 5,9,13,22,27	KRAS	exon 2-5	TP53*	exon 2-10
ERBB4	exon 3,4,6-8,15,22,27	MDM2*	exon 1-11	VHL*	exon 1-3

* Full transcript covered in this gene panel. Of the remaining genes, only regions that are frequently mutated were sequenced instead of the whole coding sequence. Amplifications were able to be detected in genes covered by at least 5 amplicons.

TABLE S7. Gene coverage of Ion AmpliSeq™ Cancer Hotspot Panel v2:

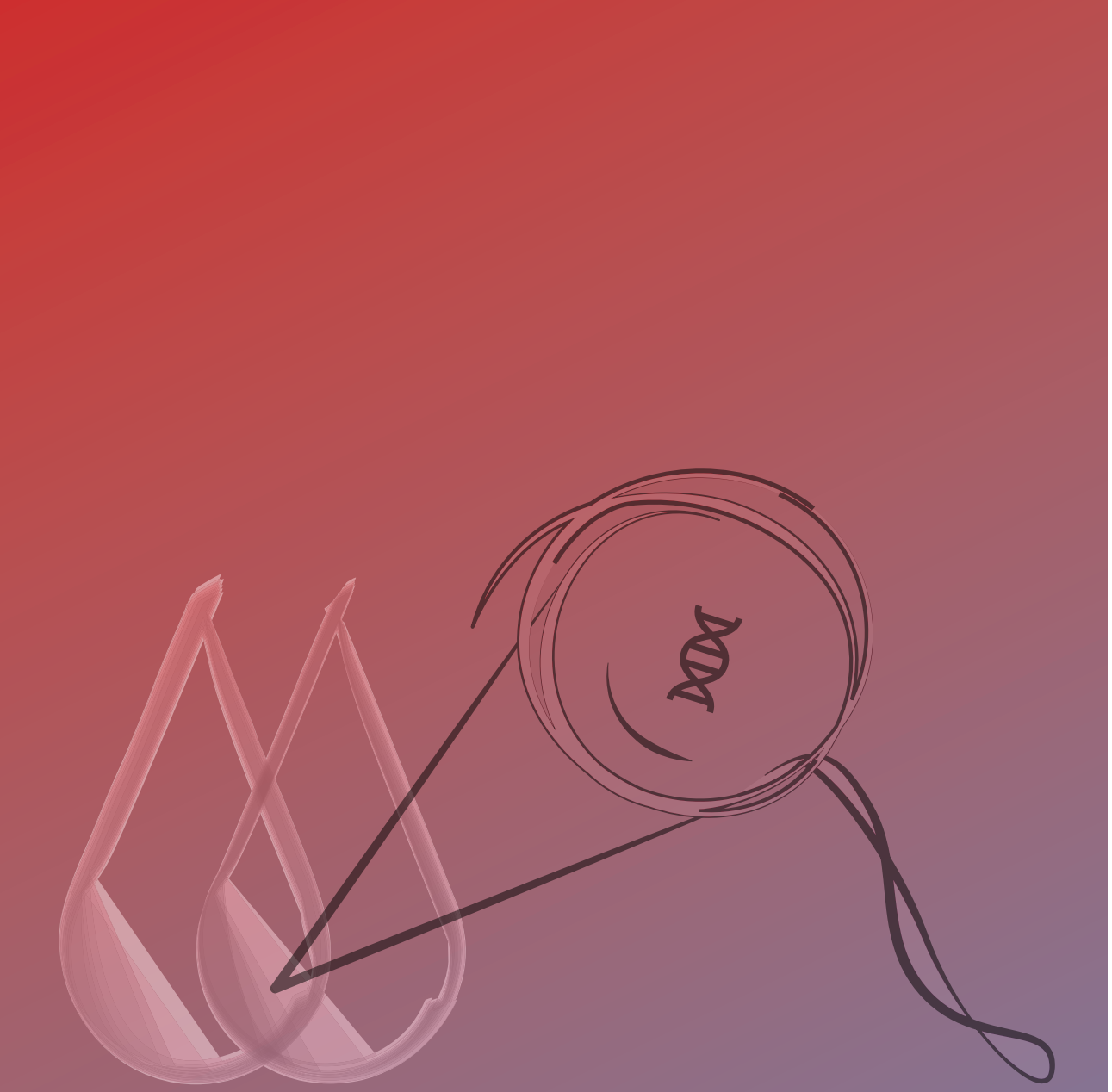
ABL1	exon 4-7	EZH2	exon 16	JAK2	exon 14	PTEN	exon 3,5-8
AKT1	exon 3, 6	FBXW 7	exon 5,8-11	JAK3	exon 4,13,16	PTPN1 1	exon 3,13
ALK	exon 23,25	FGFR 1	exon 4,7	KDR	exon 6,7,11,19,21,26,30	RB1	exon 4,6,10, 11,1,4,17
APC	exon 14	FGFR 2	exon 5,7,10	KIT	exon 2,9-11, 13-15,17,18	RET	exon 10,11,13,1,5,16
ATM	exon 8,9,12,17,26, 34,35,36,39,50,54, 55,56,59,61,63	FGFR 3	exon 7,9,14,16,18	KRAS	xon 2-4	SMAD4	exon 3-6,8-12
BRAF	exon 11,15	FLT3	exon 11,14,16,20	MET	exon 2,11,14,16,19	SMARC B1	exon 2,4,5
CDH1	exon 3,8,9	GNA1 1	exon 5	MLH1	exon 12	SMO	exon 3,5,6,9,11
CDKN 2A	exon 2	GNAS	exon 8,9	MPL	exon 10	SRC	exon 14
CSF1 R	exon 7,22	GNAQ	exon 5	NOTCH 1	exon 25,27,37	TP53	exon 3-8,10
CTNN B1	exon 3	HNF1 A	exon 3,4	NPM1	exon 11	VHL	exon 1-3
EGFR	exon 3,7,15,18-21	HRAS	exon 2,3	NRAS	exon 2-4		
ERBB 2	exon 22-24	IDH1	exon 4	PDGFR A	exon 12,14,15,18		
ERBB 4	exon 3-5,7-9, 15,23	IDH2	exon 4	PIK3CA	exon 2,5,7-10,14,19,21		

Table S8. Gene coverage of Ion AmpliSeq™ Cancer Hotspot Panel v2+:

ABL1	exon 4-7	FGFR2	exon 5,7,10	MYD88	exon 5
AKT1	exon 3,6	FGFR3	exon 7,9,14,16,18	NOTCH1	exon 25,27,37
ALK	exon 22-25		NPM1		exon 11
APC	exon 14	GNA11	exon 5	NRAS	exon 2-4
ARAF	exon 6	GNAS	exon 8,9	PDGFR A	exon 12,14,15,18
ATM	exon 8,9,12,17,26,34,35, 36,39,50,54,55,56,59,61, 63	GNAQ	exon 5	PIK3CA	exon 1,4,6,7,9,13, 18,20
BRAF	exon 11,15	HNF1A	exon 3,4	PTEN	exon 3,5-8
CALR	exon 9	HRAS	exon 2,3	PTPN11	exon 3,13
CDH1	exon 3,8,9	IDH1	exon 4	RB1	exon 4,6,10,11,14,17
CDKN2A	exon 2	IDH2	exon 4	RET	exon 10,11,13,15,16
CRAF	exon 6	JAK2	exon 14	SMAD4	exon 3-6,8-12
CSF1R	exon 7, 22	JAK3	exon 4,13,16	SMARC B1	exon 2,4,5
CTNNB1	exon 3	KDR	exon 6,7,11,19,21,26,30	SMO	exon 3,5,6,9,11
EGFR	exon 3,7,15,18-21	KIT	exon 2,9-11,13-15,17,18	SRC	exon 14
ERBB2	exon 19-24	KRAS	exon 2-4	STK11	exon 1,4,6,8
ERBB4	exon 3-5,7-9,15,23	MDM2	exon 6-9	TP53	exon 2-10
EZH2	exon 16	MET	exon 2,11,14,16,19	VHL	exon 1-3
FBXW7	exon 5,8-11		MLH1	exon 12	
FGFR1	exon 4,7		MPL	exon 10	

*This assay covers all hotspot regions, but does not give information about the complete coding sequence of depicted genes.

**This assay is not yet officially validated for the detection of amplifications



3

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

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ABSTRACT

Liquid biopsy is a minimally invasive detection method for molecular biomarkers in body fluids which may serve as a novel tool in management of head and neck cancer. The purpose of this systematic review is to outline the current status of liquid biopsy in head and neck squamous cell carcinoma (HNSCC) patients by systematically identifying and qualifying all published studies on the diagnostic or prognostic value of cell-free nucleic acids detection for posttreatment disease monitoring and/or disease outcome. A search was performed in PubMed, EMBASE, and Cochrane Library. Thirty articles met the inclusion criteria for further analysis. Study and patient characteristics, molecular analysis method and treatment or prognostic outcomes were extracted. Seventeen studies investigated circulating miRNAs in blood. Of these studies, 16 found statistically significant results for a total of 24 different candidate miRNAs for prognostication or treatment monitoring. The remaining studies investigated circulating tumor DNA by targeting somatic mutations, allelic imbalances, hypermethylation, or HPV-DNA. Of these studies, 2 found a statistically significant association between nucleic acid levels (tumor DNA targeted by allelic imbalances and HPV-DNA) in blood and/or saliva and prognostic outcome. One study found significantly different pre- and posttreatment levels of mitochondrial DNA in serum. Despite large differences among these studies in both design and results, individual results are promising and provide ground for more large-scale studies with standardized serial assessment of patient samples in the future.

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) comprise malignant tumors of the upper aerodigestive tract. Major risk factors are tobacco smoking, betel nut chewing, alcohol consumption, and human papilloma virus (HPV) infection. Oropharyngeal HNSCCs are attributable to HPV infection in 30.8% of cases worldwide [1]. Furthermore, HNSCC accounts for 3.8% of all cancer types with an estimated global incidence of approximately 600,000 patients each year [2]. This number is predicted to rise 62% by 2035, which is probably being caused by shifts in etiological and sociodemographic factors [3-6]. Current 5-year survival rates in Europe range between 25% and 60% depending on primary tumor site and stage, and improved only 3–5% in the last 2 decades [7-9]. This may partly be explained by the poor outcome after treatment of residual or recurrent disease, which in turn may be a consequence of delayed diagnosis due to difficulties in distinguishing locoregional disease from posttreatment effects (e.g. fibrosis, inflammation) on physical examination and imaging, especially during the first months of follow-up [10]. Additionally, the detection of subclinical regional or distant metastases is challenging [11, 12].Thus obviously, there is still room for improvements in posttreatment surveillance for further improving survival rates of HNSCC patients. Another strategy to improve survival rates is probably a more individualized treatment based on specific characteristics of the individual tumor.

Evolving molecular techniques are now able to target biomarkers with high accuracy in all kinds of body fluids (i.e. ‘liquid biopsy’). This enables minimally invasive profiling of tumor specific transcriptomic signatures or (epi)genetic targets. This allows a more personalized approach to cancer management by improving disease prognostication and posttreatment disease monitoring, while avoiding burdensome and invasive tissue biopsy procedures. In particular, cell-free circulating tumor DNA (ctDNA) and microRNA (miRNA) are targets of interest for various diagnostic applications, as these are derived from the tumor and shed into the bloodstream following tumor cell necrosis and/or apoptosis [13]. Cell-free ctDNA are DNA fragments of <160 bp, containing tumor-specific (epi)genetic aberrations such as mutations or methylation patterns, that can be used as diagnostic biomarkers for the early detection of tumor recurrence [14-16]. MiRNAs are small non-coding RNA fragments, which have important regulatory roles in cells by modulating transcription of mRNA through binding of three prime untranslated regions (3’ UTR). Deregulation of circulating miRNA expression patterns may be used as prognostic or diagnostic biomarkers [17]. Cell-free (circulating) HPV-DNA may be beneficial in case of HPV-positive oropharyngeal tumors for posttreatment disease monitoring [18, 19]. Molecular biomarkers also appear in saliva through ultra-filtration from blood, passive diffusion or active transport. In addition, due to its localized character, saliva can also contain DNA derived from HNSCCs through release into the upper aerodigestive tract following necrosis of apically located tumor cells [20, 21]. This makes saliva

another potential source of molecular biomarkers, as it requires an even easier, less invasive collection method than blood sampling. The use of highly sensitive techniques for the detection of molecular biomarkers is essential for all of the above mentioned purposes, since target nucleic acid levels in body fluids can be very low (e.g. 1 mutant DNA fragment per ml) [22]. Examples of available techniques for accurate quantification of targeted cell-free nucleic acids are 'beads, emulsions, amplification, and magnetics' (BEAMing), 'Safe-Sequencing System' (Safe-SeqS), 'Cancer Personalized Profiling by deep Sequencing' (Capp-Seq), 'Tagged-amplicon deep Sequencing' (Tam-Seq), and several PCR applications, while other techniques such as whole genome sequencing (WGS), whole exome sequencing (WES), and next-generation sequencing (NGS) are used for minimally invasive tumor profiling [23]. In order to outline the current status of liquid biopsy in HNSCC patients, we systematically identified and qualified all published studies investigating putative molecular targets for posttreatment disease monitoring and/or prognostic value for disease outcome by the detection of cell-free nucleic acids in blood or saliva.

MATERIAL AND METHODS

A systematic search was conducted in the PubMed, Embase, and Cochrane databases for original articles published until 20th of January 2017. A combination of 'MeSH terms' and 'free-text words' were used to search on "head and neck cancer", "(epi)genetic biomarker", "liquid biopsy" and their synonyms in title and abstract fields (Supplemental Material). Citations and references of selected articles and reviews were checked to identify potentially missed relevant studies. Two authors (J.H.G. and F.J.B.S.) independently screened all titles and abstracts of the retrieved search for selection using predefined inclusion and exclusion criteria. Subsequently, full-text of relevant studies was screened for a more detailed selection. Inclusion criteria were studies on (1) human whole-blood, plasma, serum, or saliva from histologically proven HNSCC patients, evaluating (2) cell-free nucleic acids to use as (3) biomarkers for treatment monitoring and/or disease outcome by targeting (4) (epi)genetic aberrations, RNA or HPV-DNA. Exclusion criteria were (1) no full-text available, (2) duplicate articles containing all or some of the original publication data, (3) review articles, book chapters, case reports, editorials, oral presentations, technical notes, and poster presentations, and (4) studies only focusing on patients with nasopharyngeal, esophageal, tracheal, and/or salivary gland tumors. Using a standardized data extraction form we extracted first author, year of publication, country, sample size, biomarker detection methods, tumor location, tumor HPV-status, testing matrix, (epi)genetic target and diagnostic, and associated therapeutic and/or prognostic outcome with p-values if

reported. The two authors mentioned previously performed data extraction independently, and disagreement was resolved by discussion.

RESULTS

Combined PubMed, Embase, and Cochrane searches retrieved 4,385 hits of which 3,588 unique articles remained after deduplication. After screening titles and abstracts, 183 articles remained and were retrieved in full text for formal review. Twenty-eight articles met the inclusion criteria and were eligible for further analysis [24-53]. Additionally, 15 articles were identified through reference check of the included articles, of which 2 articles met the inclusion criteria for further analysis [54, 55]. The main reasons for exclusion were duplicates and studies not investigating cell-free nucleic acids or treatment monitoring and/or prognostic outcome (Fig. 1). Of the 30 studies included, 12 investigated cell-free nucleic acids for monitoring patients treated for HNSCC, while 14 studies investigated the prognostic value of cell-free nucleic acids (either pre- or post-treatment), and 4 studies investigated both (Table 1). Study sample sizes ranged from 9 – 218, consisting of HNSCC patients with variable subsites and tumor stages. Two studies did not report tumor subsite, while 4 did not report TNM features of the tumors. Only 7 studies reported HPV-status. MiRNA was the cell-free nucleic acid most frequently investigated (17/30 studies), detected by qRT-PCR in plasma, serum, or saliva.

Nineteen of 30 included studies found a statistically significant differential expression or detectability of at least one targeted cell-free nucleic acid (Table 2 and 3). Associated prognostic outcomes were distant metastases (DM), disease- or recurrence-free survival (DFS/RFS), progression-free survival (PFS), or overall survival (OS) with median follow-up periods ranging from 6 – 52.16 months (Table 2). In most cases, upregulation of miRNAs, as well as the detection of HPV-DNA, mutant DNA and loss of heterozygosity (LOH) was associated with worse prognostic outcomes [27, 28, 32, 34, 38, 40, 51, 52]. Otherwise, downregulation of miR-9 was significantly associated with a worse outcome [53].

All 16 studies on treatment monitoring investigated surgically treated patients by analyzing and comparing samples obtained prior to treatment with samples obtained 1 – 72 weeks after treatment, with or without correlation with recurrence (Table 3). In most cases, a downregulation of miRNAs was found after treatment. One study found a posttreatment decrease of mutant mitochondrial DNA (mtDNA) in OSCC patients [31]. MiR-92, miR-139-5p, miR-375, and miR-486-5p were found to be upregulated after treatment [26, 47]. In 11 studies, no statistical significance was found in relation to treatment monitoring or prognostic outcome for any of the molecular targets investigated [24, 30, 33, 35-37, 43, 48-50, 52]. MiR-21 was the

only target that was found expressed significantly different in more than one study, being the case for both treatment monitoring and prognostication [45, 46].

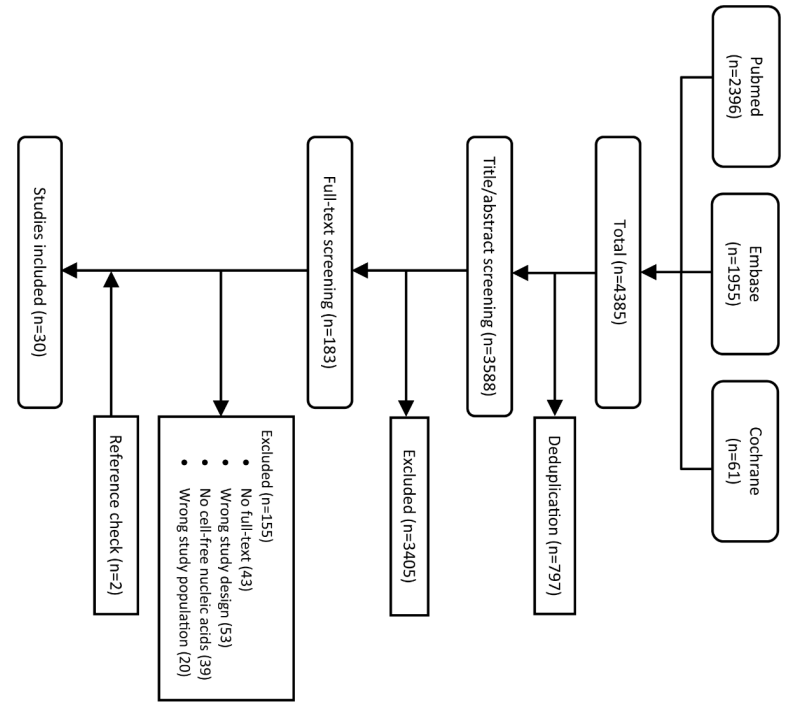

FIGURE 1. Flowchart of study inclusion

TABLE 1. Study characteristics

First Author	Year	Country	Sample size	Tumor subsite[1]	Primary tumor stage	Positive HPV-status	Matrix	Cell- free nucleic acid	Detection method[2]	Molecular target[3]	Assessed for
Nawroz	1996	USA	21	Not specified	T1: 4 T2: 4 T3: 10 T4: 3	Not reported	Serum	ctDNA	PCR	IFNA, D9S200, D9S161, D9S156, D3S1284, D3S1238, D14S50, D23S1245, CHRNB1, D17S786, FgA, DRPLA	Prognostic outcome
Sanchez-Cespedes	2000	USA	7	OSCC, OPSCC, HPSCC, LSCC	Not extractable	Not reported	Serum	ctDNA	qMSP-PCR	CDKN2A, MGMT, DAPK hypermethylation	Treatment monitoring
Nawroz-Danish	2004	USA	152	Not specified	Stage I: 6 II: 19 III: 27 IV: 87	Not reported	Serum	ctDNA	PCR	D9s161, D9s200, D3s1238, D3s1284, Chrnb1, D14s50, D9s242, D17s695, L17686, CFS1R	Prognostic outcome
Hamana	2005	Japan	64	OSCC	Stage I: 3 II: 14 III: 23 IV: 25	Not reported	Serum	ctDNA	PCR	D5S178, D9S104, IFNA, D11S910, D11S1356, D13S273, TP53, D18S46, D22S274	Prognostic outcome/ Treatment monitoring
Chuang	2008	USA	59	OSCC, OPSCC, HPSCC, LSCC	Stage I-II: 18 III-IV: 39	20	Saliva	HPV- DNA	qPCR	HPV16 E6/E7	Prognostic outcome
Kakimoto	2008	Japan	20	OSCC	T1: 5 T2: 7 T3: 3 T4: 5	Not reported	Serum	ctDNA	PCR	D2S1327, D2S206, D3S1007, D3S1079, D3S966, D21S236, D21S11, D21S1254, D21S369	Prognostic outcome
Wong	2008	China	25	OSCC	Stage I: 4 II: 14 III: 4 IV: 3	Not reported	Plasma	miRNA	qRT-PCR	**miR-184**	Treatment monitoring

TABLE 1 CONTINUED.

First Author	Year	Country	Sample size	Tumor subsite[1]	Primary tumor stage	Positive HPV-status	Matrix	Cell- free nucleic acid	Detection method[2]	Molecular target[3]	Assessed for
Liu	2010	Taiwan	43	OSCC	Stage I-II: 15 III-IV: 28	Not reported	Plasma	miRNA	qRT-PCR	**miR-31**	Treatment monitoring
Liu	2012	Taiwan	45	OSCC	Stage I-II: 21 III-IV: 24	not reported	Saliva	miRNA	qRT-PCR	**miR-31**	Treatment monitoring
Cao	2012	USA	14	OPSCC	Not extractable	14	Plasma	HPV-DNA	qPCR	HPV L1 G5+/6+, HPV16/18 E6/E7	Treatment monitoring
Hsu	2012	Taiwan	14	OSCC, OPSCC, HPSCC, LSCC	Stage I: 3 II: 1 III: 6 IV: 7	Not reported	Plasma	miRNA	qRT-PCR	Let7a, miR-21, 26b, 194, 375	Treatment monitoring
Liu	2013	Taiwan	65	OSCC	Not extractable	Not reported	Plasma	miRNA	qRT-PCR	miR-196a	Prognostic outcome
Hung	2013	Taiwan	36	OSCC	Not extractable	Not reported	Plasma	miRNA	qRT-PCR	**miR-146a**	Treatment monitoring
Summerer	2013	Germany	17	OSCC, OPSCC, HPSCC, LSCC	Not extractable	Not reported	Plasma	miRNA	qRT-PCR	miR-590-5p, 574-3p, 425-5p, 885-3p, 21-5p, 28-3p, 195-5p, 191-5p	Treatment monitoring
Liu	2014	Taiwan	57	OSCC, OPSCC	Not extractable	Not reported	Plasma	miRNA	PCR-RFLP	**miR-134**	Prognostic outcome/ Treatment monitoring
Ahn	2014	USA	54	OPSCC	Not extractable	46	Plasma, saliva	HPV-DNA	qPCR	**HPV16 E6/E7**	Prognostic outcome
Hou	2015	Japan	9	OSCC, OPSCC, HPSCC, LSCC	Stage I: 0 II: 2 III: 0 IV: 7	Not reported	Plasma	miRNA	qRT-PCR	miR-21, miR-146b, miR-155, miR-223, let-7c, miR-99a, miR- 100, miR-125b	Prognostic outcome

TABLE 1 CONTINUED.

First Author	Year	Country	Sample size	Tumor subsite[1]	Primary tumor stage	Positive HPV-status	Matrix	Cell- free nucleic acid	Detection method[2]	Molecular target[3]	Assessed for
Wang	2015	USA	9	OSCC, OPSCC, HPSCC, LSCC	Stage I: 1 II: 0 III: 4 IV: 4	5	Plasma, saliva	ctDNA	MPS	mutant FBXW7, NRAS, PIK3CA, HRAS, CDKN2A, TP53	Prognostic outcome
Summerer	2015	Germany	15	OSCC, OPSCC, HPSCC, LSCC, NPSCC, Paranasal sinus	Inextractable	6	Plasma	miRNA	qRT-PCR	miR-142-3p, miR- 186-5p, miR-195-5p, miR-374b-5p and miR-574-3p	Prognostic outcome
Uzawa	2015	Japan	60	OSCC	Stage I: 12 II: 16 III: 18 IV: 14	Not reported	Serum	mtDNA	qPCR-HRMA	**mutant ND2 and ND3**	Treatment monitoring
Dahlstrom	2015	USA	218	OPSCC	Inextractable	218	Serum	HPV- DNA	qPCR	HPV16 E6/E7	Prognostic outcome
Yilmaz	2015	Turkey	30	LSCC	T1-2: 17 T3-4: 13	Not reported	Plasma	miRNA	qRT-PCR	miR-133b, miR-221	Treatment monitoring
Xu	2016	China	85	OSCC	Inextractable	Not reported	Serum	miRNA	qRT-PCR	miR-3651, miR-483- 5, miR-494, miR-31, miR-155, miR-221, miR-15a, miR-29b, miR-142-3p, miR- 502-3p, miR-32, miR-214, miR-146b, miR-215, miR-144, miR-331-3p, miR-34a, miR-133b, miR-193b, miR-99a, miR-186, miR-125, miR-135b, miR-18a, miR-409- 3p, miR-135a	Prognostic outcome

TABLE 1 CONTINUED.

First Author	Year	Country	Sample size	Tumor subsite[1]	Primary tumor stage	Positive HPV-status	Matrix	Cell- free nucleic acid	Detection method[2]	Molecular target[3]	Assessed for
Mydlarz	2016	USA	100	OSCC, OPSCC, HPSCC, LSCC	Stage I: 11 II: 9 III: 11 IV: 69	Not reported	Serum	ctDNA	qMSP-PCR	EDNRB, p16, DCC hypermethylation	Prognostic outcome
Duz	2016	Turkey	25	OSCC	Not reported	Not reported	Saliva	miRNA	qRT-PCR	miR-33a-3p, miR- 139-5p, miR-198, RNU6b	Treatment monitoring
Liu	2016	China	63	OSCC	Stage I-III: 35 IV: 28	Not reported	Plasma	miRNA	qRT-PCR	**miR-187***	Prognostic outcome/ Treatment monitoring
Braig	2016	Germany	20	OSCC, OPSCC, HPSCC, LSCC	Stage I: 0 II: 2 III: 3 IV: 15	2	Serum	ctDNA	PCR w/ targeted sequencing	**mutant EGFR exon 12, KRAS/NRAS exons 2/3/4, HRAS exons 2/3**	Prognostic outcome
Xu	2016	China	127	LSCC	Stage I: 46 II: 35 III: 28 IV: 18	Not reported	Serum	miRNA	qRT-PCR	**miRNA-378**	Treatment monitoring/ Prognostic outcome
Yan	2016	Denmark	20	OSCC	Stage 0: 3 I: 2 II: 5 III: 4 IV: 6	Not reported	Plasma	miRNA	qRT-PCR	miR-26a-5p, miR- 148a-3p, miR-21-5p, MiR-375, miR-92b-3p, miR-486-5	Treatment monitoring
Sun	2016	China	104	OSCC	T1-2: 66 T3-4: 38	Not Reported	Serum	miRNA	qRT-PCR	**miR-9**	Prognostic outcome

[1] OSCC oral squamous cell carcinoma, OPSCC oropharyngeal squamous cell carcinoma, HPSCC hypopharyngeal squamous cell carcinoma, LSCC laryngeal squamous cell carcinoma

[2] q quantitivative, qMSP quantitative methylation-specific, qRT quantitative reverse transcription, RFLP restriction fragment length polymorphism, HRMA high-resolution melting analysis, MPS massively parallel sequencing

[3] Targets stated in bold are expressed significantly different; *Low abundant hairpin arm

TABLE 2. Cell-free nucleic acids significantly associated with prognostic outcome

Molecular target	Molecular status	Associated with	Outcome	Mdn followup period (months)	P-value	Reference
miR-9	Downregulated Downregulated	OS DFS	Worse Worse	Not reported Not reported	0.022 0.004	Sun 2016 Sun 2016
miR-28-3p	Upregulated†	PFS	Worse	13.7	0.027	Summerer 2015
miR-187*	Downregulated†	DFS	Better	46.2	0.010	Liu 2016
miR-134	Downregulated†	OS	Better		<0.001	Liu 2014
miR-191-5p	Upregulated† Upregulated†	PFS OS	Worse Worse	13.7 15.5	0.002 0.004	Summerer 2015
miR-195-5p	Upregulated†	PFS	Worse	13.7	0.029	Summerer 2015
miR-374b-5p	Upregulated Upregulated	PFS OS	Worse Worse	13.7 15.5	0.039 0.036	Summerer 2015
miR-378	Upregulated†	RFS	Worse	38.6	0.000	Xu 2016
miR-425-5p	Upregulated† Upregulated†	PFS OS	Worse Worse	13.7 15.5	0.002 0.004	Summerer 2015
miR-483-5p	Upregulated	OS	Worse	52.16	<0.001	Xu 2016
miR-574-3p	Upregulated†	PFS	Worse	13.7	0.027	Summerer 2015
HPV-16 E6/E7	Positive Positive	RFS OS	Worse Worse	49 49	0.001** 0.01**	Ahn 2014
Mutant KRAS/NRAS exons 2/3/4, HRAS exons 2/3	Positive	PFS	Worse	4.9	0.032	Braig 2016
CHRNB1, D14S50, D21S1245, D3S1238, D3S1284, D9S156, D9S161, D9S200, DRPLA	Positive	DM	Worse	Not reported	0.015	Nawroz 1996

*Low abundant hairpin arm **Significant in multivariate analysis †Posttreatment

TABLE 3. Cell-free nucleic acids significantly changed during treatment monitoring

	Posttreatment status			
Molecular target	**Molecular**	**Clinical**	**P-value**	**Reference**
miR-21	Downregulated	1 month	<0.01	Hsu 2012
	Downregulated	6 months**	0.000	Hou 2015
miR-26b	Downregulated	1 month	<0.05	Hsu 2012
miR-31	Downregulated	6 weeks	<0.0001	Liu 2010
	Downregulated	4-6 weeks	<0.0001	Liu 2012
miR-92b-3p	Upregulated	9-12 months**	<0.01	Yan 2016
miR-99a	Upregulated	6 months**	0.001	Hou 2015
miR-134	Downregulated	2 weeks	0.002	Liu 2014
miR-139-5p	Upregulated	4-6 weeks	0.020	Duz 2016
miR-146a	Downregulated	not reported	<0.01	Hung 2013
miR-184	Downregulated	not reported**	<0.001	Wong 2008
miR-187*	Downregulated	6 weeks	<0.001	Liu 2016
miR-221	Downregulated	1 month	0.020	Yilmaz 2015
miR-223	Downregulated	6 months**	0.029	Hou 2015
miR-375	Upregulated	9-12 months**	<0.01	Yan 2016
miR-378	Downregulated	6 months	<0.01	Xu 2016
miR-486-5p	Upregulated	9-12 months**	<0.01	Yan 2016
mutant ND2, ND3	Decrease	4 weeks**	<0.05	Uzawa 2015

*low abundant hairpin arm **Recurrence-free patients

DISCUSSION

Improving the early detection and prediction of recurrences in HNSCC patients is desirable, as it might allow for a more timely intervention, which in turn could result in improved disease outcome [54]. Therefore, an increasing number of studies addressing the potential use of cell-free nucleic acids as biomarkers are being conducted, as applications and possibilities in molecular diagnostics are expanding rapidly. Especially for (post)treatment monitoring, it is desirable to detect actual presence of residual or recurrent tumor tissue in order to be able to timely intervene if possible. Because cell-free DNA is cleared rapidly from the blood circulation with a half-life of about 2 hours it may reflect actual tumor burden [55]. Therefore, mutations in ctDNA may be suitable as tumor-specific biomarker. Similar results were found for circulating viral DNA [56]. Circulating miRNA levels depend on numerous variables and blood clearance is not fully clarified yet [57]. For this review, we focused on studies investigating nucleic acids, either freely circulating in blood or present in saliva, to be used as minimally invasive biomarkers for outcome prediction and/or early detection of (recurrent) HNSCC. Consequently, studies that solely validated samples for the identification of candidate targets, compared patient samples with healthy control samples, or exclusively analyzed nucleic acids extracted from cellular material isolated from body fluids, were excluded.

We identified and analyzed studies that assessed a wide variety of molecular targets in both blood and saliva from HNSCC patients. Comprising more than half of the identified studies, research on the use of circulating miRNAs for treatment monitoring or prognostication appears to be most abundant compared to other types of nucleic acids. Endogenous miRNAs are shown to be stable and detectable in stored blood plasma and can easily be characterized using universally applicable assays for quantification, facilitating the discovery and validation of putative biomarkers [58]. Thus, interrogation of many different miRNAs in parallel is desirable in order to create accurate assays for profiling of (differential) miRNA expression [59]. Therefore, validation of many different miRNAs is pivotal in order to investigate which miRNAs are significant HNSCC biomarkers in body fluids. However, the development of clinically relevant assays is challenging, because miRNAs are vastly abundant [60]. Hence, we found a relatively large amount of miRNA studies and positive results regarding the significance of candidate circulating miRNAs as biomarkers.

Cell-free HPV-DNA in body fluids from HPV-positive HNSCC patients is another emerging topic in the field of biomarker research. Its potential as a diagnostic biomarker was recently shown in a feasibility study for determining HPV-status of the primary tumor using saliva from HNSCC patients, in which a sensitivity and specificity of respectively 92.9% and 100% were found [64]. Similar results were found for circulating HPV-DNA in plasma from HNSCC patients, showing a sensitivity of 96.4% [62]. Of the 4 studies concerning HPV-DNA targeting in body fluids of HNSCC patients, only 1 study found a significant association with prognostic outcomes. Ahn et al. found worse outcomes in patients detected positive for HPV-DNA in either posttreatment saliva or plasma when used combined, but did not for posttreatment plasma when analyzed separately from saliva [52]. Interestingly, Dahlstrom et al. could neither find statistically significant predictive values for recurrence among more than 200 OPSCC patients with HPV-DNA positive pretreatment serum [48]. This supports the clinical utility of HPV-DNA in HNSCC patients as a prognostic marker in saliva, rather than its detection in blood.

While genetic alterations in tumor DNA are quite extensively under investigation as minimally invasive biomarkers for other cancer types, research on blood or saliva from HNSCC patients is sparse. Alongside somatic mutations, allelic imbalances such as microsatellite instabilities and loss of heterozygosity (LOH) are also targets that can be used as biomarkers in liquid biopsies. In a pilot study conducted by Nawroz et al., the presence of several microsatellite targets in serum was significantly associated with

a worse OS and DFS [34]. However, these preliminary findings could not be confirmed in a subsequent larger cohort study conducted by the same group [35]. Coulet et al. later investigated both microsatellite instabilities and *TP53* mutations as targets for quantifying tumor DNA in plasma from HNSCC patients, showing low rates of detection of both targets using conventional PCR [63]. More recently, van Ginkel et al. detected very low levels of mutant DNA fragments relative to wildtype DNA of down to 0.01% in plasma from HNSCC patients using highly sensitive droplet digital PCR [64]. Furthermore, Hamana et al. detected positive allelic imbalances by qPCR in all serially collected pre- and posttreatment serum samples solely from the 6 HNSCC patients that eventually developed distant metastases [65].

For the first time, Wang et al. assessed combined plasma and saliva samples on the presence of cell-free tumor DNA using Safe-SeqS, a highly sensitive digital PCR- based massively parallel sequencing method, in which they detected tumor DNA in 96% of the samples from HNSCC patients, either from saliva or plasma. In a small subset of 9 patients in whom tumor DNA was detected before treatment, additional plasma and/or saliva samples were collected during follow-up. Tumor DNA was detected 4 – 8 months after surgery in 3 of the 4 patients up to 19 months before clinical evidence of recurrent disease, while no tumor DNA was detected in the 5 patients without clinical evidence of recurrence [30]. One study aimed to investigate the underlying molecular mechanism of therapy induced resistance to cetuximab by targeting mutant EGFR and RAS in plasma from HNSCC patients [51]. They found RAS mutations in a substantial proportion of patients during cetuximab treatment, which correlated significantly with disease progression. Thus, treatment induced tumor heterogeneity was investigated, rather than absolute quantification of ctDNA for detecting actual tumor presence.

Tumor heterogeneity is considered a major point of discussion with regard to liquid biopsies and the use of circulating cell-free nucleic acids as cancer biomarkers. Subclonal populations are often inherently present in primary tumors or newly arise through therapeutic selection. These populations may develop into recurrent or metastastic outgrowths that exhibit different (epi)genetic signatures and phenotypic characteristics compared to the primary tumor they are derived from. In turn, differently altered nucleic acids are being shed into body fluids, possibly constraining their detection quantitatively and/or qualitatively. However, different techniques for cell-free nucleic acid detection are available and rapidly evolving. PCR-based techniques are becoming more powerful through multiplexing capabilities, while sequencing based techniques are becoming increasingly sensitive (e.g. ultra-deep sequencing). This would make both techniques interchangeably useful for many different clinical applications such as early detection of residual or recurrent disease, monitoring treatment response or clonal evolution, and molecular profiling or prognostication.

Thus, depending on the type and extent of molecular targets aimed to use, which method is used, and which clinical purpose it has, we think that the detection of circulating

cell-free nucleic acids eventually will be of clinical value in head and neck cancer patients, despite the challenge of tumor heterogeneity and clonal evolution.

LIMITATIONS

The studies we identified are highly heterogeneous in both design and results: sample sizes were often small, or an even smaller subset of patient samples was available for analysis. Furthermore, tumor subsites and stages of included patients varied heavily between studies: in some studies all HNSCC patients were included, while other studies only focused on one specific subsite, mostly oral cancer. Some overlap was present regarding the investigated molecular targets in different studies. And while some studies focused on a single target, others assessed predetermined expression profiles of multiple miRNAs. Similar for ctDNA, targets were mostly assessed non- specifically by analysis of multiple targets altogether for one particular outcome. Variable outcome parameters and follow-up periods were assessed in prognostic studies, lacking uniformity in assessing the prognostic value of different molecular targets.

Differences in study results could also have been caused by measurement errors and batch effects, i.e. technical heterogeneity between experiments [66]. Although the PCR techniques were used quite consistently for the appropriate targets among the different studies, other procedures during sample workup and analysis could have attributed to inconsistencies in study results. For instance, blood sample workup (collection and processing), assay specificity and sensitivity, control sample quality and quantity, and post-processing could all affect the end results. This particularly accounts for (high- throughput) miRNA expression profiling. Altogether, these limitations disputed the pooling of available data for further analysis.

CONCLUSION

We provided an overview of relevant findings from all studies investigating cell-free nucleic acids in body fluids to be used as prognostic or diagnostic biomarkers for prediction of disease outcome or treatment monitoring of HNSCC patients. While almost all studies that investigated circulating miRNAs yielded statistically significant results regarding its use for either treatment monitoring or prognostication, studies that investigated tumor DNA for the same clinical purposes are less common and showed only trends towards significance.

Research on nucleic acids in saliva for prognostication and treatment monitoring of HNSCC patients is still scarce, and its results are very preliminary. In general, substantial heterogeneity on multiple levels exists in this field of research, probably leading to inconsistent results among studies. Therefore, more large-scale prospective studies are needed, in which samples are retrieved serially using standardized protocols for sample workup until molecular analysis. Nonetheless, we expect the (combined) use of these potential biomarkers eventually to provide a more personalized approach to clinical decision-making during the management of HNSCC patients in the future, which would ultimately lead to improved disease outcome.

Conflict of interest

All authors declare no conflict of interest.

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REFERENCES

1. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. Lancet Glob Health. 2016;4:e609-16.
2. Gupta B, Johnson NW, Kumar N. Global epidemiology of head and neck cancers: a continuing challenge. Oncology. 2016;91:13-23.
3. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. CA Cancer J Clin. 2017;67:51-64.
4. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2013;31:4550-9.
5. Purkayastha M, McMahon AD, Gibson J, Conway DI. Trends of oral cavity, oropharyngeal and laryngeal cancer incidence in Scotland (1975-2012) - A socioeconomic perspective. Oral oncology. 2016;61:70-5.
6. van Dijk BA, Brands MT, Geurts SM, Merkx MA, Roodenburg JL. Trends in oral cavity cancer incidence, mortality, survival and treatment in the Netherlands. International journal of cancer Journal international du cancer. 2016;139:574-83.
7. De Angelis R, Sant M, Coleman MP, Francisci S, Baili P, Pierannunzio D, et al. Cancer survival in Europe 1999-2007 by country and age: results of EUROCARE--5-a population-based study. The Lancet Oncology. 2014;15:23-34.
8. 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;51:2130-43.
9. Braakhuis BJ, Leemans CR, Visser O. Incidence and survival trends of head and neck squamous cell carcinoma in the Netherlands between 1989 and 2011. Oral oncology. 2014;50:670-5.
10. Leung AS, Rath TJ, Hughes MA, Kim S, Branstetter BFt. Optimal timing of first posttreatment FDG PET/CT in head and neck squamous cell carcinoma. Head & neck. 2016;38 Suppl 1:E853-8.
11. Muller J, Hullner M, Strobel K, Huber GF, Burger IA, Haerle SK. The value of (18) F-FDG-PET/CT imaging in oral cavity cancer patients following surgical reconstruction. Laryngoscope. 2015;125:1861-8.
12. Sohn B, Koh YW, Kang WJ, Lee JH, Shin NY, Kim J. Is there an additive value of 18F-FDG PET-CT to CT/MRI for detecting nodal metastasis in oropharyngeal squamous cell carcinoma patients with palpably negative neck? Acta Radiol. 2016;57:1352-9.
13. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nature Reviews Cancer. 2011;11:426-37.
14. Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M. Origins, structures, and functions of circulating DNA in oncology. Cancer metastasis reviews. 2016;35:347-76.
15. Wan JC, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nature reviews Cancer. 2017;17:223-38.
16. Lehmann-Werman R, Neiman D, Zemmour H, Moss J, Magenheim J, Vaknin- Dembinsky A, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. Proceedings of the National Academy

of Sciences of the United States of America. 2016;113:E1826-E34.
17. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO molecular medicine. 2012;4:143- 59.
18. Yu KH, Lo YM, Tse GM, Chan KC, Chan AB, Chow KC, et al. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nonnasopharyngeal head and neck carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10:1726-32.
19. Capone RB, Pai SI, Koch WM, Gillison ML, Danish HN, Westra WH, et al. Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6:4171-5.
20. Wang X, Kaczor-Urbanowicz KE, Wong DT. Salivary biomarkers in cancer detection. Medical oncology (Northwood, London, England). 2017;34:7.
21. Kaczor-Urbanowicz KE, Martin Carreras-Presas C, Aro K, Tu M, Garcia-Godoy F, Wong DT. Saliva diagnostics - Current views and directions. Experimental biology and medicine (Maywood, NJ). 2017;242:459-72.
22. 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. Science translational medicine. 2014;6:224ra24.
23. Heitzer E, Ulz P, Geigl JB. Circulating Tumor DNA as a Liquid Biopsy for Cancer. Clin Chem. 2015;61:112-23.
24. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer research. 2000;60:892-5.
25. Yilmaz SS, Guzel E, Karatas OF, Yilmaz M, Creighton CJ, Ozen M. MiR-221 as a pre- and postoperative plasma biomarker for larynx cancer patients. The Laryngoscope. 2015;125:E377-81.
26. Yan Y, Wang X, Veno MT, Bakholdt V, Sorensen JA, Krogdahl A, et al. Circulating miRNAs as biomarkers for oral squamous cell carcinoma recurrence in operated patients. Oncotarget. 2016;0.
27. Xu Y, Lin Y-P, Yang D, Zhang G, Zhou H-F. Expression of serum microRNA-378 and its clinical significance in laryngeal squamous cell carcinoma. European review for medical and pharmacological sciences. 2016;20:5137-42.
28. Xu H, Yang Y, Zhao H, Yang X, Luo Y, Ren Y, et al. Serum miR-483-5p: a novel diagnostic and prognostic biomarker for patients with oral squamous cell carcinoma. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2016;37:447-53.
29. Wong T-S, Liu X-B, Wong BY-H, Ng RW-M, Yuen AP-W, Wei WI. Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14:2588-92.
30. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Science translational medicine. 2015;7:293ra104- 293ra104.
31. Uzawa K, Kasamatsu A, Baba T, Kimura Y, Nakashima D, Higo M, et al. Quantitative detection of circulating tumor-derived mitochondrial NADH subunit variants as a potential prognostic biomarker for oral cancer. International journal of oncology. 2015;47:1077-83.
32. Summerer I, Unger K, Braselmann H, Schuettrumpf L, Maihoefer C, Baumeister P, et al. Circulating microRNAs as prognostic therapy biomarkers in head and neck cancer patients. British journal of cancer. 2015;113:76-82.
33. Summerer I, Niyazi M, Unger K, Pitea A, Zangen V, Hess J, et al. Changes in circulating microRNAs after radiochemotherapy in head and neck cancer patients. Radiation oncology (London, England). 2013;8:296-.
34. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nature medicine. 1996;2:1035-7.
35. Nawroz-Danish H, Eisenberger CF, Yoo GH, Wu L, Koch W, Black C, et al. Microsatellite analysis of serum DNA in patients with head and neck cancer. International journal of cancer. 2004;111:96-100.
36. Mydlarz WK, Hennessey PT, Wang H, Carvalho AL, Califano JA. Serum biomarkers for detection of head and neck squamous cell carcinoma. Head & neck. 2016;38:9-14.
37. Liu C-J, Tsai M-M, Tu H-F, Lui M-T, Cheng H-W, Lin S-C. miR-196a overexpression and miR-196a2 gene polymorphism are prognostic predictors of oral carcinomas. Annals of surgical oncology. 2013;20:S406-14.
38. Liu C-J, Shen WG, Peng S-Y, Cheng H-W, Kao S-Y, Lin S-C, et al. miR-134 induces oncogenicity and metastasis in head and neck carcinoma through targeting WWOX gene. International journal of cancer. 2014;134:811-21.
39. 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- 24.
40. Liu C-J, Lin J-S, Cheng H-W, Hsu Y-H, Cheng C-Y, Lin S-C. Plasma miR-187* is a potential biomarker for oral carcinoma. Clinical oral investigations. 2016;0.
41. Liu C-J, Kao S-Y, Tu H-F, Tsai M-M, Chang K-W, Lin S-C. Increase of microRNA miR-31 level in plasma could be a potential marker of oral cancer. Oral diseases. 2010;16:360-4.
42. Lerner C, Wemmert S, Bochen F, Kulas P, Linxweiler M, Hasenfus A, et al. Characterization of miR-146a and miR-155 in blood, tissue and cell lines of head and neck squamous cell carcinoma patients and their impact on cell proliferation and migration. Journal of cancer research and clinical oncology. 2016;142:757-66.
43. Kakimoto Y, Yamamoto N, Shibahara T. Microsatellite analysis of serum DNA in patients with oral squamous cell carcinoma. Oncology reports. 2008;20:1195-200.
44. Hung P-S, Liu C-J, Chou C-S, Kao S-Y, Yang C-C, Chang K-W, et al. miR-146a enhances the oncogenicity of oral carcinoma by concomitant targeting of the IRAK1, TRAF6 and NUMB genes. PloS one. 2013;8:e79926-e.
45. Hsu C-M, Lin P-M, Wang Y-M, Chen Z-J, Lin S-F, Yang M-Y. Circulating miRNA is a novel marker for head and neck squamous cell carcinoma. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2012;33:1933-42.
46. Hou B, Ishinaga H, Midorikawa K, Shah SA, Nakamura S, Hiraku Y, et al. Circulating microRNAs as novel prognosis biomarkers for head and neck squamous cell carcinoma. Cancer biology & therapy. 2015;16:1042-6.
47. Duz MB, Karatas OF, Guzel E, Turgut NF, Yilmaz M, Creighton CJ, et al. Identification of miR-139-5p as a saliva biomarker for tongue squamous cell carcinoma: a pilot study. Cellular oncology (Dordrecht). 2016;39:187-93.
48. Dahlstrom KR, Li G, Hussey CS, Vo JT, Wei Q, Zhao C, et al. Circulating human papillomavirus DNA as a marker for disease extent and recurrence among patients with oropharyngeal cancer. Cancer. 2015;121:3455-64.
49. Chuang AY, Chuang TC, Chang S, Zhou S, Begum S, Westra WH, et al. Presence of HPV DNA in convalescent salivary rinses is an adverse prognostic marker in head and neck squamous cell carcinoma. Oral oncology. 2008;44:915-9.

50. Cao H, Banh A, Kwok S, Shi X, Wu S, Krakow T, et al. Quantitation of human papillomavirus DNA in plasma of oropharyngeal carcinoma patients. International journal of radiation oncology, biology, physics. 2012;82:e351-8.
51. Braig F, Voigtlaender M, Schieferdecker A, Busch C-J, Laban S, Grob T, 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;7:42988-95.
52. Ahn SM, Chan JYK, Zhang Z, Wang H, Khan Z, Bishop JA, et al. Saliva and plasma quantitative polymerase chain reaction-based detection and surveillance of human papillomavirus-related head and neck cancer. JAMA Otolaryngology - Head and Neck Surgery. 2014;140:846-54.
53. Sun L, Liu L, Fu H, Wang Q, Shi Y. Association of decreased expression of serum miR-9 with poor prognosis of oral squamous cell carcinoma patients. Medical science monitor : international medical journal of experimental and clinical research. 2016;22:289-94.
54. Chang JH, Wu CC, Yuan KS, Wu ATH, Wu SY. Locoregionally recurrent head and neck squamous cell carcinoma: incidence, survival, prognostic factors, and treatment outcomes. Oncotarget. 2017.
55. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nature Medicine. 2008;14:985-90.
56. To EW, Chan KC, Leung SF, Chan LY, To KF, Chan AT, et al. Rapid clearance of plasma Epstein-Barr virus DNA after surgical treatment of nasopharyngeal carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9:3254-9.
57. Becker N, Lockwood CM. Pre-analytical variables in miRNA analysis. Clinical biochemistry. 2013;46:861-8.
58. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proceedings of the National Academy of Sciences of the United States of America. 2008;105:10513-8.
59. He YQ, Lin JJ, Kong DL, Huang MY, Xu CK, Kim TK, et al. Current state of circulating microRNAs as cancer biomarkers. Clin Chem. 2015;61:1138-55.
60. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet. 2012;13:358-69.
61. Chai RC, Lim Y, Frazer IH, Wan YX, Perry C, Jones L, et al. A pilot study to compare the detection of HPV-16 biomarkers in salivary oral rinses with tumour p16(INK4a) expression in head and neck squamous cell carcinoma patients. Bmc Cancer. 2016;16.
62. Mazurek AM, Rutkowski T, Fiszer-Kierzkowska A, Malusecka E, Skladowski K. Assessment of the total cfDNA and HPV16/18 detection in plasma samples of head and neck squamous cell carcinoma patients. Oral oncology. 2016;54:36-41.
63. Coulet F, Blons H, Cabelguenne A, Lecomte T, Lacourreye O, Brasnu D, et al. Detection of plasma tumor DNA in head and neck squamous cell carcinoma by microsatellite typing and p53 mutation analysis. Cancer research. 2000;60:707-11.
64. van Ginkel JH, Huibers MMH, van Es RJJ, de Bree R, Willems SM. Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. BMC Cancer. 2017;17:428.
65. 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. British journal of cancer. 2005;92:2181-4.
66. Goh WW, Wang W, Wong L. Why batch effects matter in omics data, and how to avoid them. Trends Biotechnol. 2017;35:498-507.

SUPPLEMENTARY DATA

Search date: 20-01-2017 Pubmed:

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#3
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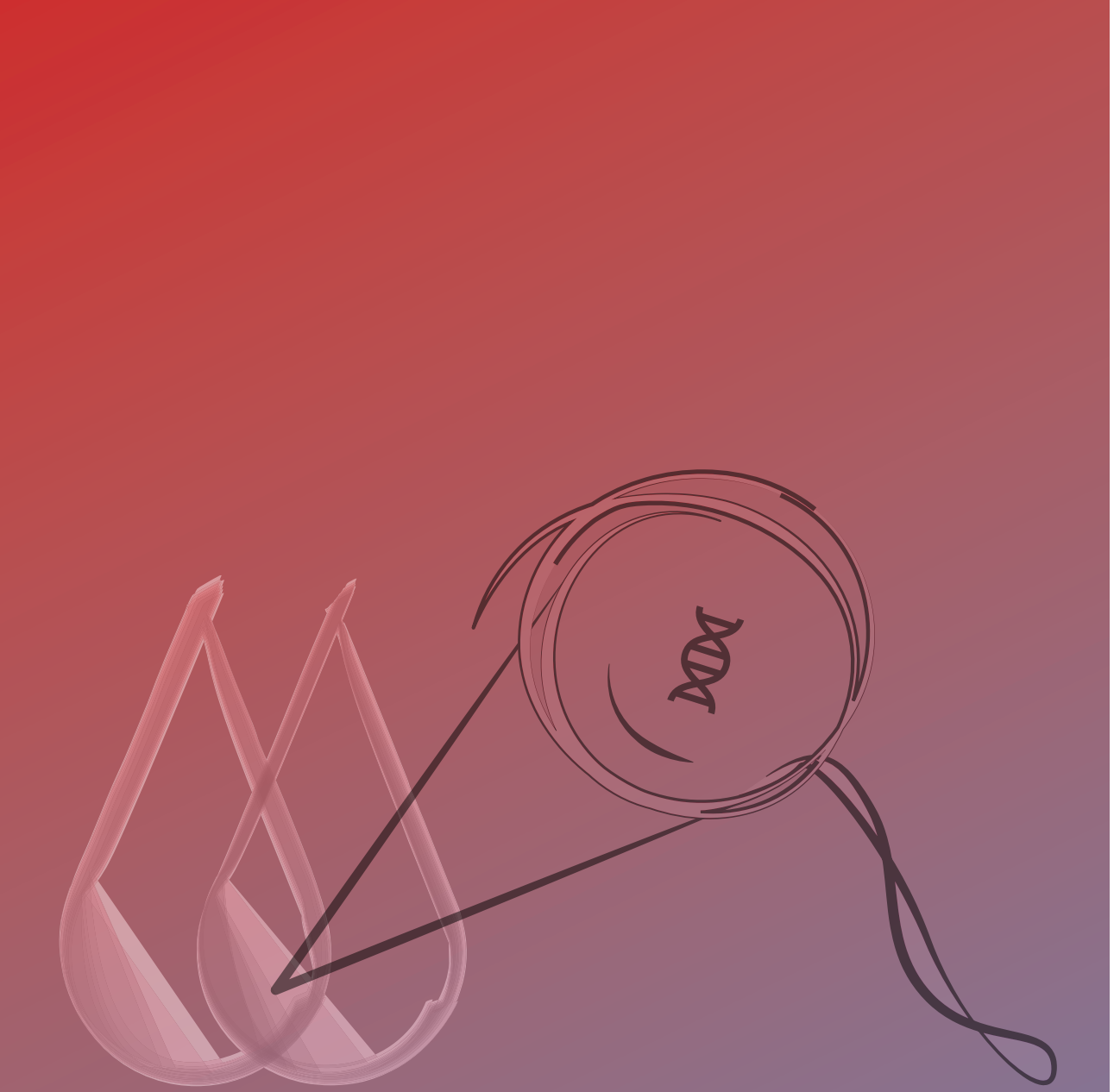

4

Droplet digital PCR for detection and quantification of rare circulating tumor DNA in plasma of head and neck cancer patients

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ABSTRACT

Background

During posttreatment surveillance of head and neck cancer patients, imaging is insufficiently accurate for the early detection of relapsing disease. Free circulating tumor DNA (ctDNA) may serve as a novel biomarker for monitoring tumor burden during posttreatment surveillance of these patients. In this exploratory study, we investigated whether low level ctDNA in plasma of head and neck cancer patients can be detected using Droplet Digital PCR (ddPCR).

Methods

TP53 Mutations were determined in surgically resected primary tumor samples from 6 patients with high stage (II-IV), moderate to poorly differentiated head and neck squamous cell carcinoma (HNSCC). Subsequently, mutation specific ddPCR assays were designed. Pretreatment plasma samples from these patients were examined on the presence of ctDNA by ddPCR using the mutation-specific assays. The ddPCR results were evaluated alongside clinicopathological data.

Results

In all cases, plasma samples were found positive for targeted *TP53* mutations in varying degrees (absolute quantification of 2.2 – 422 mutational copies/ml plasma). Mutations were detected in wild-type *TP53* background templates of 7,667 – 156,667 copies/ml plasma, yielding fractional abundances of down to 0.01%.

Conclusions

Our results show that detection of tumor specific *TP53* mutations in low level ctDNA from HNSCC patients using ddPCR is technically feasible and provide ground for future research on ctDNA quantification for the use of diagnostic biomarkers in the posttreatment surveillance of HNSCC patients.

BACKGROUND

Monitoring tumor response during posttreatment surveillance of head and neck cancer patients heavily relies on clinical examination supported by endoscopy and/or imaging (e.g. computerized tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography (PET)). However, early detection of recurrent disease is challenging due to lymph nodal micrometastases and radiation or surgery induced fibrosis and inflammation, obscuring residual or recurrent tumor tissue [1-3]. Accurate and timely detection of locoregional metastases and recurrent disease is pivotal as survival rates rapidly decline with late detection and delayed salvage surgery [4, 5]. With recent developments in molecular diagnostics, the use of (blood-based) genetic biomarkers is growing in a wide variety of cancer types [6]. Cell free circulating tumor DNA (ctDNA), released into the bloodstream by apoptotic and necrotic tumor cells, harbor tumor-specific mutations [7]. These mutations can be detected in blood plasma from cancer patients by blood sampling, also known as “liquid biopsy” [8]. For head and neck cancer, research has been focused mainly on actionable oncogenic mutations such as *PIK3CA* and *HRAS*, hot-spot *TP53* mutations, and HPV-related biomarkers to use as prognosticators or predictors for establishing and adjusting targeted therapy [9-12]. For similar purposes, transcriptional and epigenetic changes are studied substantially [13-15]. For the early detection of recurrent disease, early driver mutations in HNSCC such as *TP53* mutations would be favorable to use as biomarkers, as these are likely to occur consistently throughout clonal evolution [16, 17], and are found to be most frequent and concordant in recurrent and metastatic HPV-negative tumors compared to mutations in other genes [18-22]. By targeting and quantifying early driver mutations in ctDNA, tumor burden could be monitored after treatment, facilitating earlier detection of asymptomatic residual and/or recurrent disease. Previous studies showed correlations between ctDNA levels and tumor dynamics during posttreatment monitoring in patients with various types of cancer [23-26]. However, accurate detection of ctDNA in plasma is challenging, because ctDNA concentrations can be very low. This could greatly impair reliable and valid measurement of tumor dynamics. Highly sensitive Droplet Digital PCR (ddPCR) facilitates detection and quantification of low levels of ctDNA by partitioning DNA samples into 20,000 water-in-oil droplets [27]. In this exploratory study, we investigated whether detection and quantification of ctDNA in plasma from several head and neck squamous cell carcinoma (HNSCC) patients using ddPCR is technically feasible.

METHODS

Patients and samples

Six patients (median age 60.5 [42 – 77] years) with histologically confirmed HPV- negative HNSCC were selected retrospectively for analysis of archived primary tumor samples and presurgically obtained blood samples. Patient selection was based on TNM stage (stage II or higher) and availability of blood plasma samples in our biobank. Additional clinicopathological and radiological data were collected from hospital charts of selected patients (Table 1; Figure 1).

Sample workup

All primary tumor samples were acquired from formalin fixed paraffin embedded (FFPE) incisional or excisional biopsy specimens, microscopically containing >30% tumor cells. In order to reveal *TP53* mutation status of primary tumor samples, targeted next-generation sequencing (NGS) was performed using the Ion Torrent™ PGM platform (Thermo Fisher Scientific, Waltham, MA, USA), as previously described [28]. NGS was based on the Cancer Hotspot Panel v2+ (Thermo Fisher Scientific, Waltham, MA, USA), covering *TP53* exons 2 – 10 [29]. All blood samples were collected in 10 ml K_2EDTA blood collection tubes (BD Vacutainer, Franklin Lakes, NJ, USA). Prior to archiving, centrifugation took place for 10 min at 800 g (Rotina 380, Hettich, Germany), after which supernatant plasma was aliquoted in 1 ml portions and stored at -80°C until DNA isolation. Storage time of patient FFPE and corresponding plasma samples varied from 4 months to 9 years.

Table 1. Summary of patient and tumor characteristics

Patient ID	Sex	Smoking (pack years)	Alcohol (units/ day)	Biopsy type	TNM-stage	Tumor site[a]	Differen-tiation grade	Max diameter primary tumor (mm)	Growth type[b]	Vascular invasion
P1	M	0	8	Excisional	T4aN1M0	OSCC	Moderate	40	NS	No
P2	M	0	0	Excisional	T4aN2cM0	OSCC	Poor	72	NS	Yes
P3	F	0	0	Excisional	T2N0Mx	OSCC	Moderate	32	Unknown	Yes
P4	M	Unknown	1	Excisional	T4aN2bM0	OSCC	Moderate	46	S	No
P5	M	49	12	Excisional	T4aN1M0	OSCC	Moderate/ poor	37	Unknown	No
P6	F	42	2	Incisional	T3N2cM0	OPSCC	Unknown	13	N/A	No

a OSCC = Oral Squamous Cell Carcinoma; OPSCC = Oropharyngeal Squamous Cell Carcinoma b NS = Non Spiculated; S = Spiculated

Plasma samples were thawed and DNA was immediately isolated from 2 ml of plasma using QIAamp Circulating Nucleic Acid (NA) kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated plasma samples were eluted in 50 µl elution buffer as provided with the kit and stored at 4°C until ddPCR analysis. Positive control samples, containing both wild-type (WT) and mutant (MT) DNA, were created for all patients by isolating tumor DNA from the primary tumor FFPE samples using COBAS DNA Sample Preparation Kit (Roche, Basel, Switzerland) according to manufacturer's instructions. After quantity measurement of isolated DNA samples with a Qubit fluorometer using the dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific), cfDNA was diluted to 10 ng/ul using purified water. For each assay, no template controls (NTC) were used to control for environmental contamination, and wild-type-only (WT-only) samples were used in order to estimate false-positive rates. Five WT-only samples were created by isolating plasma DNA from anonymous healthy individuals using the QIAamp Circulating NA kit.

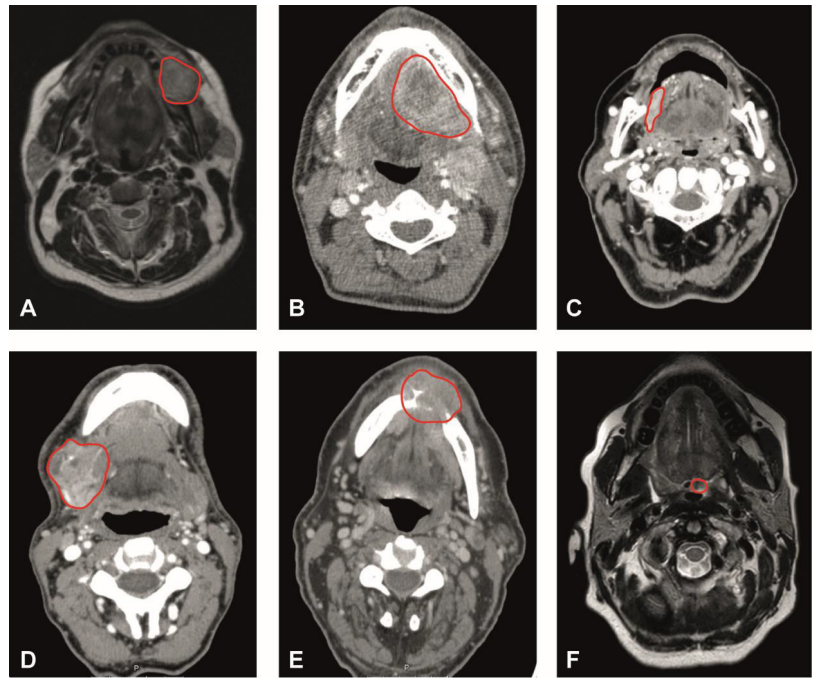

FIGURE 1. Primary tumors of 6 patients encircled in red. (A) Axial T1 MRI image of a tumor of the left mandible of patient 1. (B) Axial ceCT image of a tumor in the floor of mouth of patient 2. (C) Axial ceCT image of a tumor in the right lateral tongue of patient 3. (D) Axial ceCT image of a tumor in right mandible/floor of mouth/tongue of patient 4. (E) Axial ceCT image of a tumor in the floor of mouth in patient 5. (F) Axial T1 MRI image of tumor in left mid tongue base of patient 6. ceCT, contrast enhanced computed tomography

ddPCR

The plasma samples from all 6 patients were analyzed for *TP53* point mutations, identified in the primary tumor tissue by NGS. MT and WT *TP53* sequences were used as DNA template for designing ddPCR (Bio-Rad Laboratories, Hercules, CA, USA) assays following the MIQE guidelines (Supplementary Table S1) [30]. DdPCR reaction volumes of 22 µl were prepared, consisting of 13 µl mastermix (11 µl Supermix for Probes [no deoxyuridine triphosphate], 1 µl of primer/probe mix for both MT and WT *TP53*), and 9 µl cfDNA sample of patient plasma. The NTCs contained 9 µl of purified water instead of cfDNA sample. The WT-only samples contained 1 – 7 ul of cfDNA. From the PCR reaction mixture, 20 µl was used for droplet generation. Droplet Digital PCR was performed using the QX200 ddPCR system according to manufacturer's instructions (Bio-Rad Laboratories). QuantaSoft v1.7.4.0917 (Bio-Rad Laboratories) software was used for data analysis.

Prior to plasma sample testing, thermal gradient experiments were performed on FFPE samples in order to determine optimal amplification conditions during thermal cycling for each assay independently. Based on clearest separation of negative and positive droplet clusters, thermal cycling conditions for all 6 assays were set at 95°C for 10 min (1 cycle), 94 °C for 30 s and 55°C for 60 s (55 cycles), and infinite hold at 12°C. To ensure experiment quality, wells with total droplet counts of less than 10,000 would be considered invalid and excluded from analysis. The positive control samples were used to verify assay performance and facilitate thresholding in fluorescence values. Additionally, positive control samples were validated by comparing the fractional abundance (FA) in FFPE samples to NGS mutation frequencies. False-positive rate estimation was determined by performing 5 experiments for each assay using the WT-only samples, where total amounts of detected MT-positive droplets determined thresholds above which positive droplets in patient samples were to be considered as true positive.

Post-analysis

For each patient, plasma was analyzed in duplicate. Therefore, PCR results of patients samples were based on the mean of estimated target DNA concentrations (copies/µl) in merged wells, automatically calculated by manufacturer software. Correction for false positivity was performed by virtually subtracting the amount of MT-false-positive droplets from the amount of MT-positive droplets detected in the patients sample with the corresponding assays. Subsequently, absolute sample concentrations were (re)calculated as described in Supplementary Data (Equation S1). Relative quantification was defined as the FA of MT to total (WT + MT) copies.

RESULTS

Assay validation

In all six patients, *TP53* mutations were detected in FFPE by both NGS and ddPCR (Supplementary Table S1 and Figure S1). FA of MT copies ranged from 6.1 – 71.7% in positive control samples, compared to NGS MT template percentages of 7 – 70%. False-positive rate estimation was necessary to determine aspecific MT signal (Supplementary Table S2). One MT-false-positive droplet was detected in the WT-only sample experimental series for assay 1 and 3, establishing a true positivity threshold of >1 MT-positive droplet for these assays. For the remaining assays, no MT-false- positive droplets were detected in the WT-only samples. WT-false-positive droplets for all used assays in NTCs ranged from 0 – 10 droplets (Supplementary Table S2 and Figure S2). No MT-positive droplets were detected in any of the NTC samples.

ctDNA quantification

The amount of ctDNA was quantified and analyzed in blood plasma samples from all 6 patients (Table 2). MT copies of *TP53* were detected in plasma samples from all patients (Figure 2A), ranging from 0.04 – 7.60 copies/µl ddPCR mix and 1 – 181 MT- positive droplets in merged wells (Figure 2B). When corrected for MT-false-positive droplets, plasma ctDNA concentrations ranged from 2.2 – 422 copies/ml plasma (Figure 3A). MT copies were detected in WT backgrounds of 138 – 2821 copies/µl, yielding FA of MT copies of 0.01 – 5.2% (Figure 3B).

TABLE 2. Absolute and relative quantifications of MT and WT DNA in plasma samples from HNSCC patients

Sample ID	MT DNA concentration			WT DNA concentration		FA$_{mut}$
	Sample (copies/µl)	Samplecorr (copies/µl)	Plasma (copies/ml)	Reaction (copies/µl)	Plasma (copies/ml)	
P1	0.47	0.43	24	315	17 500	0.13%
P2	7.60	7.60	422	138	7 667	5.50%
P3	0.17	0.16	8.9	158	8 778	0.10%
P4	1.79	1.79	99	2 821	156 667	0.06%
P5	0.37	0.37	21	380	21 167	0.10%
P6	0.04	0.04	2.2	397	22 056	0.01%

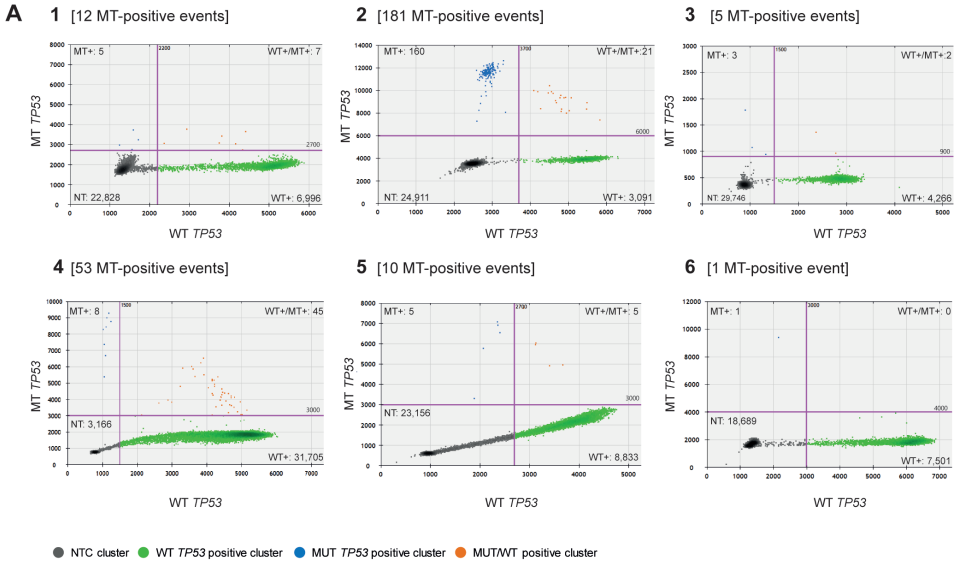

FIGURE 2. 2D-plots and amount of MT-positive droplets of ddPCR results of all 6 patients. (A) All diagrams (1-6) represent merged ddPCR results of duplicates of corresponding patient samples (1-6), showing MT-positive droplet clusters (blue dots), negative droplet clusters (dark grey dots), and MT/WT-positive droplets (orange dots). The green dots represent WT-positive droplets, proving existence of cfDNA in the samples and satisfactory ddPCR conditions. Purple lines are manually placed thresholds for distinguishing positive and negative droplets, which were set at fluorescence values based on ddPCR results of FFPE samples. (B) The amount of MT-positive and negative droplets based on thresholds as placed in 2D-plots in (A).

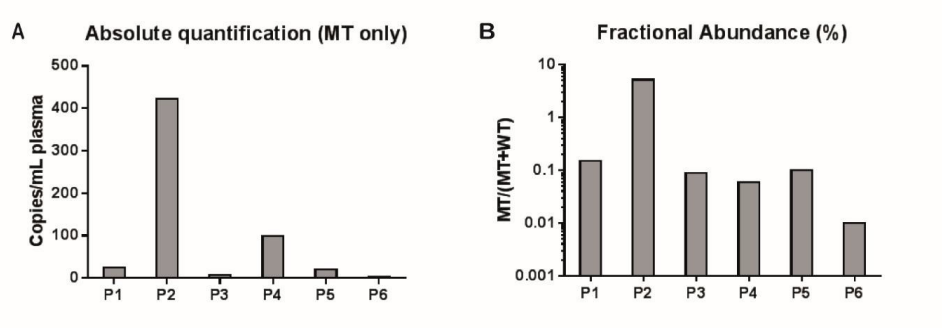

FIGURE 3. DdPCR results of patients (P1-P6) showing absolute quantification of ctDNA concentrations in plasma (A), and log-scaled fractional abundances of MT copies from total amount of MT and WT copies as corrected for total DNA input (B).

DISCUSSION

Our study shows that quantification of rare target mutations in ctDNA in plasma from HNSCC patients using ddPCR is technically feasible. Highly sensitive detection methods like digital PCR are needed in order to detect rare MT targets within high concentrations of WT background [31]. WT background size (i.e. concentration of WT cfDNA) can strongly vary over time for each patient individually, depending on multiple factors. For instance, patient's physical status (e.g. inflammation, post-traumatic, post-exercise, chronic illness), as well as pre-analytical technical procedures (e.g. white blood cell lysis caused by whole blood transportation and processing) appear to affect cfDNA concentrations [32-35]. Increased cfDNA concentration causes dilution of ctDNA, which could lower the accuracy of rare MT fragment detection. Therefore, pre-analytical steps should be most optimally in lowering background DNA; e.g. blood plasma instead of serum is preferred as source for ctDNA, as the amount of cfDNA in serum can be 2 – 4 times higher than that in plasma [36]. It has been shown for various applications that ddPCR is capable of rare target DNA quantification with higher precision and accuracy compared to quantitative PCR [27, 37-39]. Although we did not perform quantitative PCR we found relative quantification measurements of MT copies down to 0.01%. This falls within the potential dynamic range for absolute quantification of rare target DNA within a 100,000-fold of WT background as previously demonstrated [40, 41]. Similar quantification results were reported in a study where *TP53* mutations were identified in plasma using another PCR-based detection method in 88% of HPV-negative HNSCC patients (n=22) with MT fractions varying between 0.016 – 2.9% [42]. We also found large variability in MT quantification measurements among patient samples.

This is consistent with previous mutation analysis of blood samples from HNSCC patients, in which MT *TP53* fragments of 0 – 1500 per 5 ml plasma were targeted and detected by conventional PCR [43].

Variances in detected MT copies among patients can be the result of various (pre) analytical deficiencies and technical errors like plasma sample contamination from the environment. Furthermore, decreased DNA concentration due to prolonged storage, poor sample quality, subsampling during whole blood retrieval and/or centrifugation, inefficient DNA isolation from plasma samples, poor droplet handling leading to shredding or coalition of droplets, instrument artifacts, intrinsic PCR errors caused by PCR inhibition and/or minor mismatches between primer/probes and target molecules can all affect PCR results [44, 45].

During ddPCR post-analysis, manual threshold determination and stochastic sampling errors could directly lead to over- or underestimation of target copies, resulting in inaccurate quantification of results [46]. Furthermore, we know from previous validation experiences that fluorescence values of positive droplet clusters can vary inter-experiment, while assessing DNA samples derived from the same individual and using identical ddPCR assays. The same holds true for ddPCR experiments on DNA samples derived from different plasma matrices and/or volumes, containing different PCR inhibitors [47]. These points concerning post-analysis need to be addressed in order to implement ddPCR for ctDNA quantification into clinical practice. Therefore each assay and each sample should be analyzed individually. Although we used FFPE for positive control samples for threshold placement and plasma from different individuals for false-positive rate estimation, samples were patient specific and of similar matrix of DNA source, respectively. In this way, plasma DNA composition from the patients was mimicked most realistically. Moreover, the alternative of using (spiked) series of artificially synthesized DNA oligonucleotides for creating control samples can provoke overestimation of PCR targets due to the high purity of these solutions. Eventually, interpretation of ddPCR results depends on the accuracy of ctDNA quantification which is determined by false positive rate estimation.

Several biological factors could affect ctDNA concentration. Especially tumor volume is of interest as it may reflect tumor burden and actual disease status through correlation with ctDNA concentration. Simultaneously, tumor characteristics such as histological grade, localization, growth pattern, growth rate, and degree of vascularization possibly complicate reliable monitoring of tumor burden by ctDNA quantification, as these factors might affect ctDNA release into the bloodstream all differently [44, 48]. However, in a series of 117 patients with primary HNSCC, no significant correlation was found between gender, tumor stage, site, and plasma ctDNA concentration detected by touchdown PCR [49]. Interestingly, in our study, the highest amount of ctDNA was detected in plasma from the patient that harbored the largest tumor diameter of all 6 included patients. This tumor also had a poor histological differentiation grade with vascular invasion. At the other end, the lowest amount of ctDNA

was detected in plasma from the patient with the smallest tumor diameter and without vascular invasion. However, we studied and compared plasma samples retrieved at one time point from a rather small group of high-stage HNSCC patients with presumably greater tumor burden and plasma ctDNA concentrations.

Therefore, serial ctDNA quantification in clinical patients diagnosed with primary HNSCC of all stages is needed to clarify its significance for posttreatment disease monitoring and the possible advantages of its specific application with respect to early tumor detection in relation to current clinical diagnostics [50]. Tumor heterogeneity could further complicate monitoring tumor burden through ctDNA detection, because intratumoral heterogeneity of the primary tumor induces branched tumor evolution of subclonal populations harboring different molecular alterations [51]. This could lead to increased clonal heterogeneity between primary tumor and matched metastatic or recurrent tumors, risking mistargeting of ctDNA. However, as early driver *TP53* mutations show high concordance between primary and recurrent and/or metastatic tumors, these may hold promise as most reliable targets for ctDNA detection and for early tumor detection of HNSCC recurrences [21].

CONCLUSION

The detection of tumor specific *TP53* mutations in ctDNA from HNSCC using a ddPCR is technically feasible and provide ground for further research on ctDNA quantification to be used as a diagnostic biomarker in the posttreatment surveillance of HNSCC patients.

List of abbreviations

ctDNA, circulating tumor DNA; ddPCR, droplet digital polymerase chain reaction; HNSCC, head and neck squamous cell carcinoma; MRI, magnetic resonance imaging; CT, computer tomography; PET, positron emission tomography; HPV, human papilloma virus; FFPE, formalin fixed paraffin embedded; NGS, next-generation sequencing; MT, mutant; WT, wild type; FA, fractional abundance

DECLARATIONS

Ethics approval and consent to participate

All patients were treated in University Medical Center Utrecht. According to Dutch national ethical guidelines, no ethical approval to use leftover material for scientific purposes is

required, as the use of anonymous leftover material is part of the treatment agreement with patients at the University Medical Center Utrecht [52]. Administrative permission was received from the hospital for accessing the hospital medical records for research purposes.

Consent for publication

According to Dutch legislation, no informed consent to publish clinical information is required as only anonymous data was used [53].

Availability of data and material

Supporting data can be found in Additional File 1. Raw data generated and analyzed during this study is electronically available upon request by contacting the corresponding author of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JG, MH and SW conceived and designed the study. MH, SW, RB, and RE were involved in drafting and revising the manuscript critically for important intellectual content. RB and RE collected and provided biomaterials and clinicopathological data. JG and MH carried out the experiments. JG and MH analyzed and interpreted the data. JG wrote the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. Ferlito A, Partridge M, Brennan J, Hamakawa H. Lymph node micrometastases in head and neck cancer: a review. Acta oto-laryngologica. 2001;121:660-5.
2. de Bree R, van der Putten L, Brouwer J, Castelijns JA, Hoekstra OS, Leemans CR. Detection of locoregional recurrent head and neck cancer after (chemo)radiotherapy using modern imaging. Oral oncology. 2009;45:386-93.
3. Muller J, Hullner M, Strobel K, Huber GF, Burger IA, Haerle SK. The value of (18) F-FDG-PET/CT imaging in oral cavity cancer patients following surgical reconstruction. Laryngoscope. 2015;125:1861-8.
4. Gleber-Netto FO, Braakhuis BJ, Triantafyllou A, Takes RP, Kelner N, Rodrigo JP, et al. Molecular events in relapsed oral squamous cell carcinoma: Recurrence vs. secondary primary tumor. Oral oncology. 2015;51:738-44.
5. Yom SS, Machtay M, Biel MA, Sinard RJ, El-Naggar AK, Weber RS, et al. Survival impact of planned restaging and early surgical salvage following definitive chemoradiation for locally advanced squamous cell carcinomas of the oropharynx and hypopharynx. Am J Clin Oncol-Canc. 2005;28:385-92.
6. Kalia M. Biomarkers for personalized oncology: recent advances and future challenges. Metabolism. 2015;64:S16-21.
7. 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 research. 2001;61:1659-65.
8. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014;32:579-86.
9. Nemunaitis J, Clayman G, Agarwala SS, Hrushesky W, Wells JR, Moore C, et al. Biomarkers Predict p53 Gene Therapy Efficacy in Recurrent Squamous Cell Carcinoma of the Head and Neck. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15:7719-25.
10. Lui VW, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. Cancer discovery. 2013;3:761-9.
11. Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsague X, Laporte L, et al. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. The Lancet Oncology. 2014;15:1319-31.
12. Koole K, Brunen D, van Kempen PM, Noorlag R, de Bree R, Lieftink C, et al. FGFR1 Is a Potential Prognostic Biomarker and Therapeutic Target in Head and Neck Squamous Cell Carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016;22:3884-93.
13. Arantes LMRB, de Carvalho AC, Melendez ME, Carvalho AL, Goloni-Bertollo EM. Methylation as a biomarker for head and neck cancer. Oral oncology. 2014;50:587-92.
14. Noorlag R, van Kempen PMW, Moelans CB, de Jong R, Blok LER, Koole R, et al. Promoter hypermethylation using 24-gene array in early head and neck cancer Better outcome in oral than in oropharyngeal cancer. Epigenetics. 2014;9:1220-7.
15. Zhang M, Zhao LJ, Liang WQ, Mao ZP. Identification of microRNAs as diagnostic biomarkers in screening of

head and neck cancer: a meta-analysis. Genet Mol Res. 2015;14:16562-76.

16. Boyle JO, Hakim J, Koch W, van der Riet P, Hruban RH, Roa RA, et al. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer research. 1993;53:4477-80.
17. Nees M, Homann N, Discher H, Andl T, Enders C, Herold-Mende C, et al. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. Cancer research. 1993;53:4189-96.
18. Hedberg ML, Goh G, Chiosea SI, Bauman JE, Freilino ML, Zeng Y, et al. Genetic landscape of metastatic and recurrent head and neck squamous cell carcinoma. The Journal of clinical investigation. 2016;126:169-80.
19. Morris LG, Chandramohan R, West L, Zehir A, Chakravarty D, Pfister DG, et al. The Molecular Landscape of Recurrent and Metastatic Head and Neck Cancers: Insights From a Precision Oncology Sequencing Platform. JAMA Oncol. 2016.
20. Hiley C, de Bruin EC, McGranahan N, Swanton C. Deciphering intratumor heterogeneity and temporal acquisition of driver events to refine precision medicine. Genome biology. 2014;15:453.
21. van Ginkel JH, de Leng WW, de Bree R, van Es RJ, Willems SM. Targeted sequencing reveals TP53 as a potential diagnostic biomarker in the post-treatment surveillance of head and neck cancer. Oncotarget. 2016.
22. Seiwert TY, Zuo ZX, Keck MK, Khattri A, Pedamallu CS, Stricker T, et al. Integrative and Comparative Genomic Analysis of HPV-Positive and HPV-Negative Head and Neck Squamous Cell Carcinomas. Clinical Cancer Research. 2015;21:632- 41.
23. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;14:985–90.
24. Dawson SJ, Tsui DWY, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer. New Engl J Med. 2013;368:1199-209.
25. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget. 2015;6:42008-18.
26. Reinert T, Scholer LV, Thomsen R, Tobiasen H, Vang S, Nordentoft I, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2015.
27. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods. 2013;10:1003-5.
28. de Leng WW, Gadellaa-van Hooijdonk CG, Barendregt-Smouter FA, Koudijs MJ, Nijman I, Hinrichs JW, et al. Targeted Next Generation Sequencing as a Reliable Diagnostic Assay for the Detection of Somatic Mutations in Tumours Using Minimal DNA Amounts from Formalin Fixed Paraffin Embedded Material. PloS one. 2016;11:e0149405.
29. Hoogstraat M, Hinrichs JW, Besselink NJ, Radersma-van Loon JH, de Voijs CM, Peeters T, et al. Simultaneous detection of clinically relevant mutations and amplifications for routine cancer pathology. The Journal of molecular diagnostics : JMD. 2015;17:10-8.
30. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, et al. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin Chem. 2013;59:892-902.
31. Qin Z, Ljubimov VA, Zhou C, Tong Y, Liang J. Cell-free circulating tumor DNA in cancer. Chin J Cancer. 2016;35:36.
32. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer--a survey. Biochimica et biophysica acta. 2007;1775:181-232.
33. Rothwell DG, Smith N, Morris D, Leong HS, Li YY, Hollebecque A, et al. Genetic profiling of tumours using both circulating free DNA and circulating tumour cells isolated from the same preserved whole blood sample. Molecular oncology. 2016;10:566-74.
34. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: Preanalytical considerations. Clinica chimica acta; international journal of clinical chemistry. 2013;424:222-30.
35. Swarup V, Rajeswari MR. Circulating (cell-free) nucleic acids - A promising, non- invasive tool for early detection of several human diseases. Febs Lett. 2007;581:795- 9.
36. Jung M, Klotzek S, Lewandowski M, Fleischhacker M, Jung K. Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem. 2003;49:1028-9.
37. Wang P, Jing F, Li G, Wu Z, Cheng Z, Zhang J, et al. Absolute quantification of lung cancer related microRNA by droplet digital PCR. Biosens Bioelectron. 2015;74:836-42.
38. Tang H, Cai Q, Li H, Hu P. Comparison of droplet digital PCR to real-time PCR for quantification of hepatitis B virus DNA. Biosci Biotechnol Biochem. 2016:1-6.
39. Ruelle J, Yfantis V, Duquenne A, Goubau P. Validation of an ultrasensitive digital droplet PCR assay for HIV-2 plasma RNA quantification. J Int Aids Soc. 2014;17:19675.
40. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical chemistry. 2011;83:8604-10.
41. Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, et al. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Analytical chemistry. 2012;84:1003-11.
42. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Science translational medicine. 2015;7:293ra104.
43. 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. Science translational medicine. 2014;6:224ra24.
44. Ignatiadis M, Lee M, Jeffrey SS. Circulating Tumor Cells and Circulating Tumor DNA: Challenges and Opportunities on the Path to Clinical Utility. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21:4786-800.
45. Sozzi G, Roz L, Conte D, Mariani L, Andriani F, Verderio P, et al. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. Journal of the National Cancer Institute. 2005;97:1848-50.
46. Trypsteen W, Vynck M, De Neve J, Bonczkowski P, Kiselinova M, Malatinkova E, et al. ddpcRquant: threshold determination for single channel droplet digital PCR experiments. Analytical and bioanalytical chemistry. 2015;407:5827-34.
47. Devonshire AS, Whale AS, Gutteridge A, Jones G, Cowen S, Foy CA, et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. Analytical

and bioanalytical chemistry. 2014;406:6499-512.

48. Nygaard AD, Holdgaard PC, Spindler KLG, Pallisgaard N, Jakobsen A. The correlation between cell-free DNA and tumour burden was estimated by PET/CT in patients with advanced NSCLC. British journal of cancer. 2014;110:363-8.

49. Coulet F, Blons H, Cabelguenne A, Lecomte T, Lacourreye O, Brasnu D, et al. Detection of plasma tumor DNA in head and neck squamous cell carcinoma by microsatellite typing and p53 mutation analysis. Cancer research. 2000;60:707-11.

50. Heitzer E, Ulz P, Geigl JB. Circulating Tumor DNA as a Liquid Biopsy for Cancer. Clin Chem. 2015;61:112-23.

51. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature. 2013;501:338-45.

52. van Diest PJ. For and against - No consent should be needed for using leftover body material for scientific purposes - For. Brit Med J. 2002;325:648-9.

53. Netherlands. Medical Research Involving Human Subjects Act. Bull Med Ethics. 1999;No. 152:13-8.

TABLE S1. NGS data and PCR assays

Patient ID	Gene	Mutation	Mutation effect	Mutant protein	NGS mutation frequency	ddPCR Mutation FA	PCR assay	MIQE Context [wt/mut]	Amplicon length
1	*TP53*	c.920-1G>T	Splice acceptor	Unknown	19%	22.4%	COSM95740	AGACCAAGGGTGCAGTTATGCCTCAGAT TCACTTTTATCACCTTTCCTTGCCTCTTT CCTA[G/T]CACTGCCCAACAACACCAGCT CCTCTCCCCAGCCAAAGAAGAAACCACT GGATGGAGAATA	68 bp
2	*TP53*	c.700T>G	Missense	p.T234A	44%	34.3%	COSM146344	ACTGGCCTCATCTTGGGCCTGTGTTATC TCCTAGGTTGGCTCTGACTGTACCACCA TCCAC[T/G]ACAACTACATGTGTAACAGT TCCTGCATGGGCGGCATGAACCGGAGG CCCATCCTCACCAT	64 bp
3	*TP53*	c.526T>C	Missense	p.C176A	7%	6.1%	COSM44948	CCGCGTCCGCGCCATGGCCATCTACAA GCAGTCACAGCACATGACGGAGGTTGT GAGGCGC[T/C]GCCCCCACCATGAGCGC TGCTCAGATAGCGATGGTGAGCAGCTG GGGCTGGAGAGACGACA	65 bp
4	*TP53*	c.625A>T	Stop gained	p.I255P	20%	42.5%	COSM43651	CAACTACATGTGTAACAGTTCCTGCATG GGCGGCATGAACCGGAGGCCCATCCTC ACCATC[A/T]TCACACTGGAAGACTCCAG GTCAGGAGCCACTTGCCACCCTGCACA CTGGCCTGCTGTGCC	64 bp
5	*TP53*	c.763A>T	Missense	p.A209*	50%	46.6%	COSM11290	GGCCCCTCCTCAGCATCTTATCCGAGTG GAAGGAAATTTGCGTGTGGAGTATTTGG ATGAC[A/T]GAAACACTTTTCGACATAGT GTGGTGGTGCCCTATGAGCCGCCTGAG GTCTGGTTTGCAAC	65 bp
6	*TP53*	c.1015G>T	Missense	p.E339*	70%	71.7%	COSM214290	TGTGTATATACTTACTTCTCCCCCTCCTC TGTTGCTGCAGATCCGTGGGCGTGAGC GCTTC[G/T]AGATGTTCCGAGAGCTGAAT GAGGCCTTGGAACTCAAGGATGCCCAG GCTGGGAAGGAGCC	65 bp

EQUATION S1. Absolute quantification was determined by calculating the number of copies of target DNA per ml plasma using the sample concentrations:

$$C = -\ln\left(\frac{N_{neg}}{N}\right) / V_{droplet}$$

C = sample concentration (copies/µl)
N_{neg} = number of negative droplets
N = total number of droplets
$V_{droplet}$ = volume of droplet (0.85 nl)

$$PC = C \cdot RV \cdot \frac{EV}{TV} / PV$$

PC = plasma concentration (copies/ml)
C = sample concentration (copies/µl)
RV = PCR reaction volume (20 µl)
EV = volume in which cfDNA was eluted (50 µl);
TV = volume of cfDNA added to the PCR reaction (9 µl)
PV = volume of plasma used for cfDNA extraction (2 ml)

TABLE S2. Assay validation: limit of detection (LOD)

	Sample	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Fluorescence amplitude (MUT/WT)		2700/2200	6000/3700	900/1500	3000/1500	3000/2700	4000/3000
# Positive droplets in WT-only samples (MUT/WT)	1	0/2794	0/2762	1/3632	0/3138	0/3212	N/A
	2	1/12892	0/16515	0/15167	0/17775	0/16314	0/18230
	3	3/836	N/A	0/921	0/834	0/1035	0/814
	4	0/943	0/656	0/1072	0/855	0/822	0/733
	5	0/518	0/555	0/794	0/651	0/580	0/696
MT-false-positive droplets		4	0	1	0	0	0
Mean false-positive concentration (copies/µl)		0.064	0	0.012	0	0	0
# Positive droplets in NTC samples (MUT/WT)	1	0/1	0/4	0/1	0/3	0/0	0/0
	2	0/0	N/A	0/0	0/2	0/0	0/0
	3	0/1	0/2	0/1	0/4	0/0	0/0
	4	0/2	0/0	0/5	0/1	0/0	0/1
Mean positive droplets		0/1	0/2	0/2	0/3	0/0	0/0

N/A = not analyzed (total droplet count < 10,000)

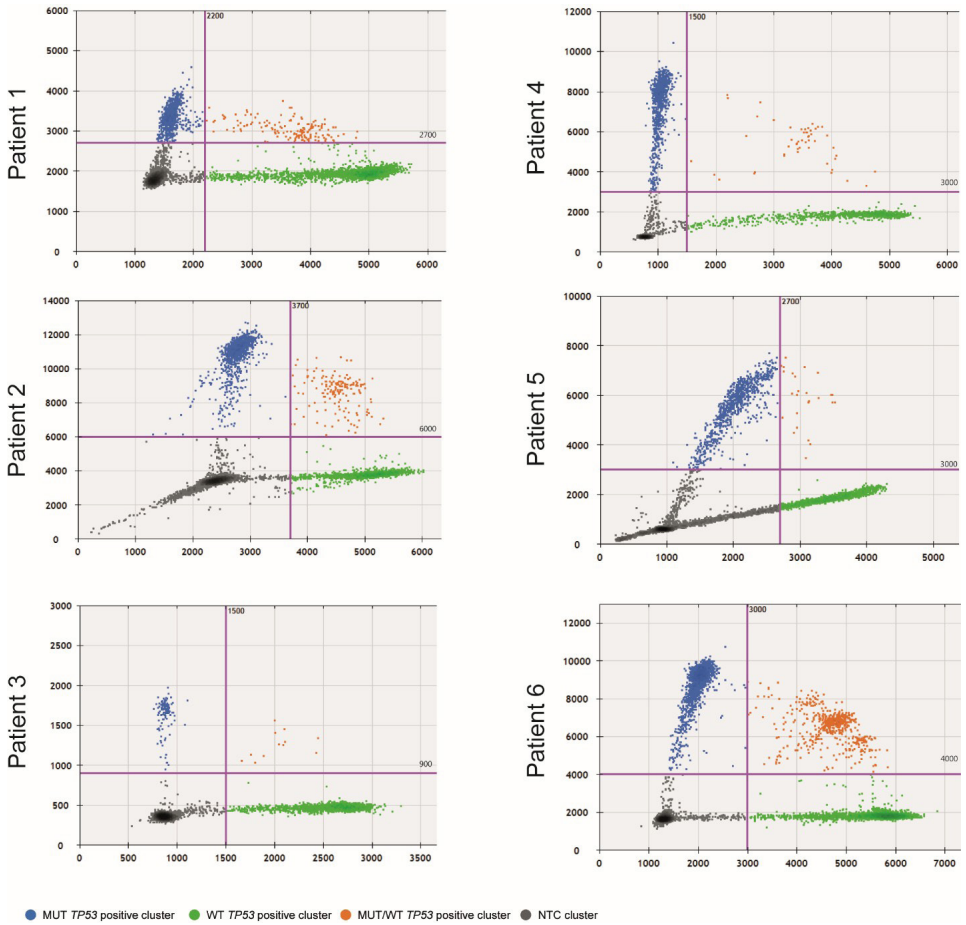


FIGURE S1. DdPCR results of 6 different MT *TP53* assays on positive control (FFPE) samples of all 6 patients are shown. The MT-positive clusters (blue dots) and MT/WT-positive clusters (orange dots) are clearly separated from the negative droplet clusters (dark grey dots) and WT-positive droplet clusters. Thresholds are placed manually.

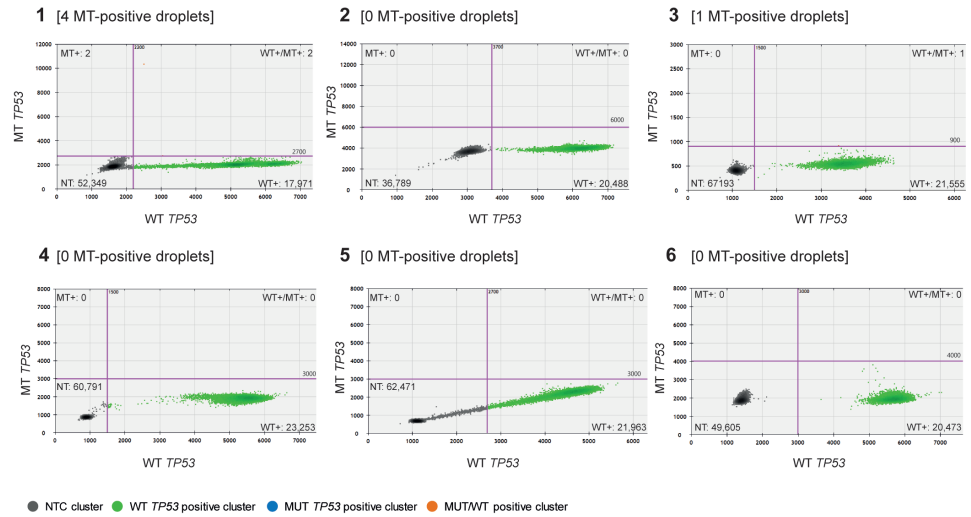


FIGURE S2. 2D-plots with the amounts of droplets of ddPCR results in healthy individuals using assay 1-6. All threshold are placed using exact values as derived from the 2D-plots in Supplementary Fig S1. The plots represent merged results of plasma samples from 4-5 different healthy individuals for each assay. MT+ MT-positive droplets, WT+ WT-positive droplets, MT+/WT+ MT/WT-positive droplets, NT No template droplets

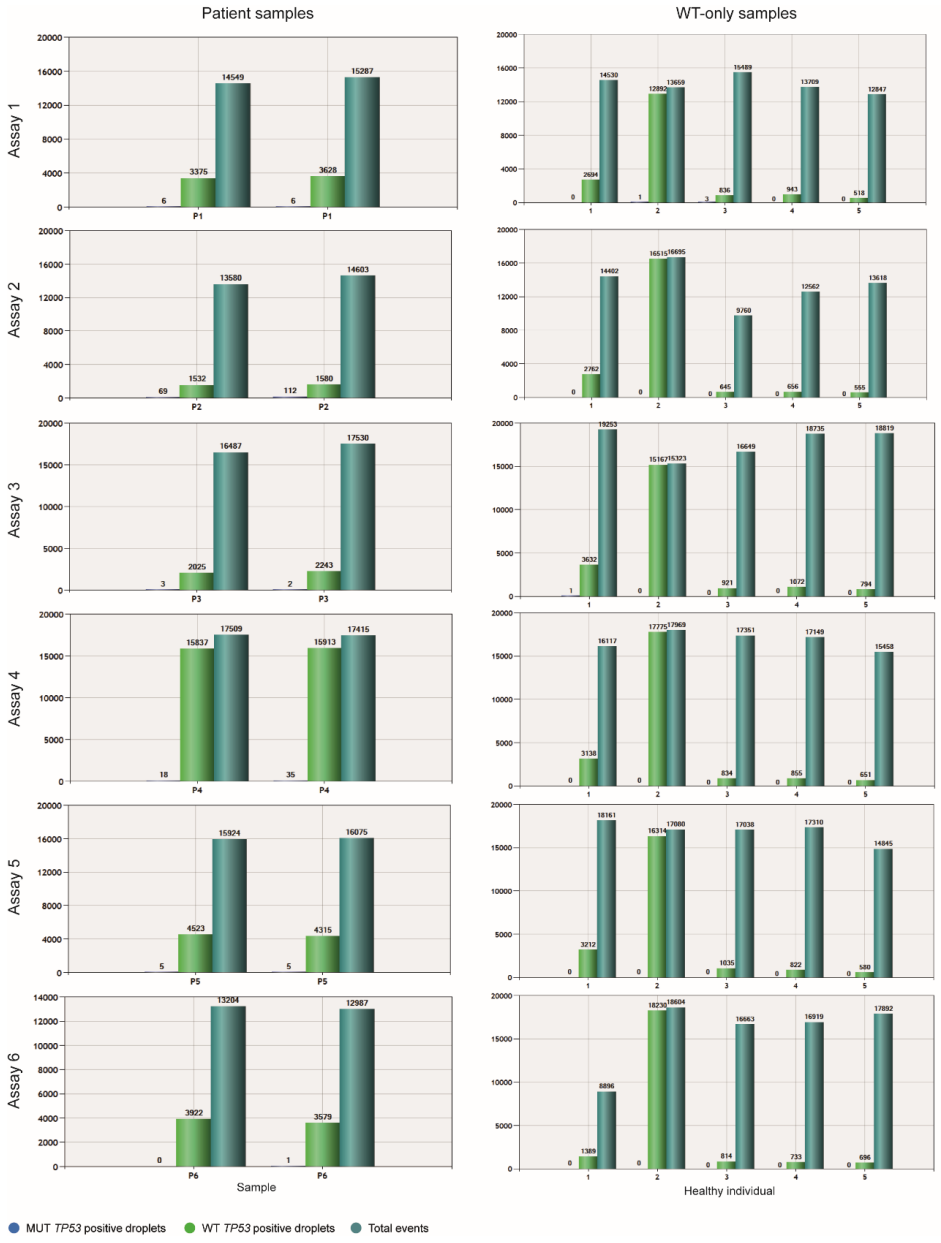


FIGURE S3. DdPCR results for all 6 patients side-by-side with the WT-only samples from healthy individuals. All patient samples are shown in duplicate. In order to estimate the false positive rate for patient samples, plasma samples from 5 different healthy individuals were used. In the samples from healthy individuals 3 and 1 used during validation of assay 2 and assay 6, less than 10,000 droplets were detected. Therefore, these results were excluded from false positive estimation for the corresponding assays.

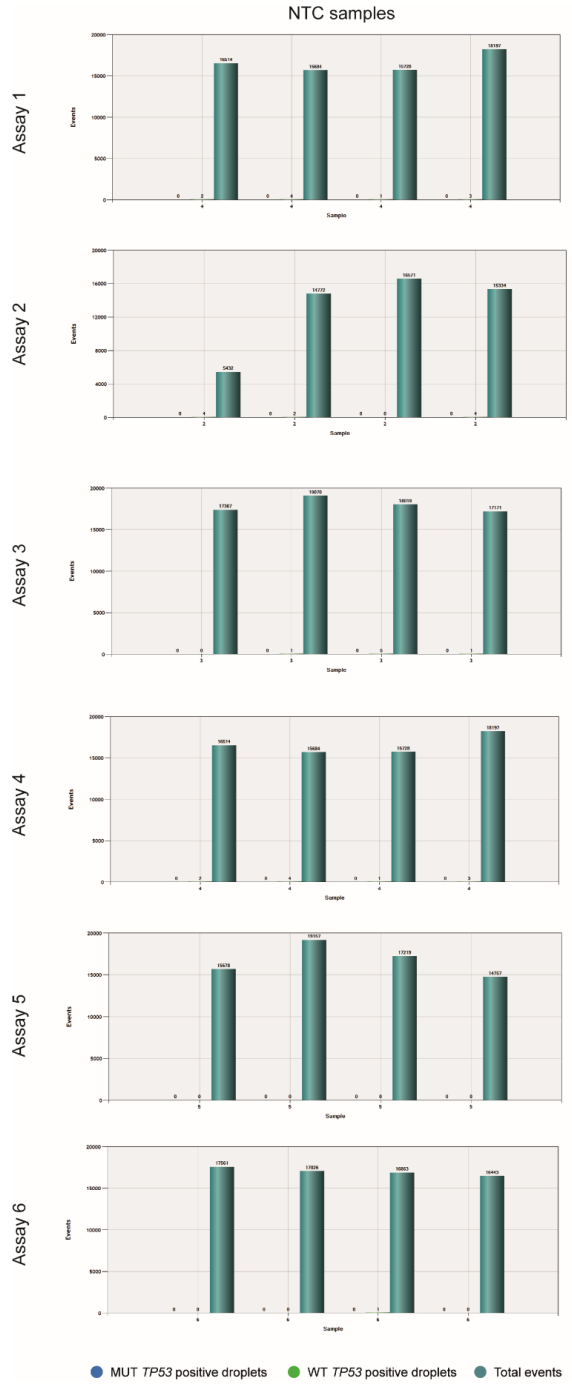
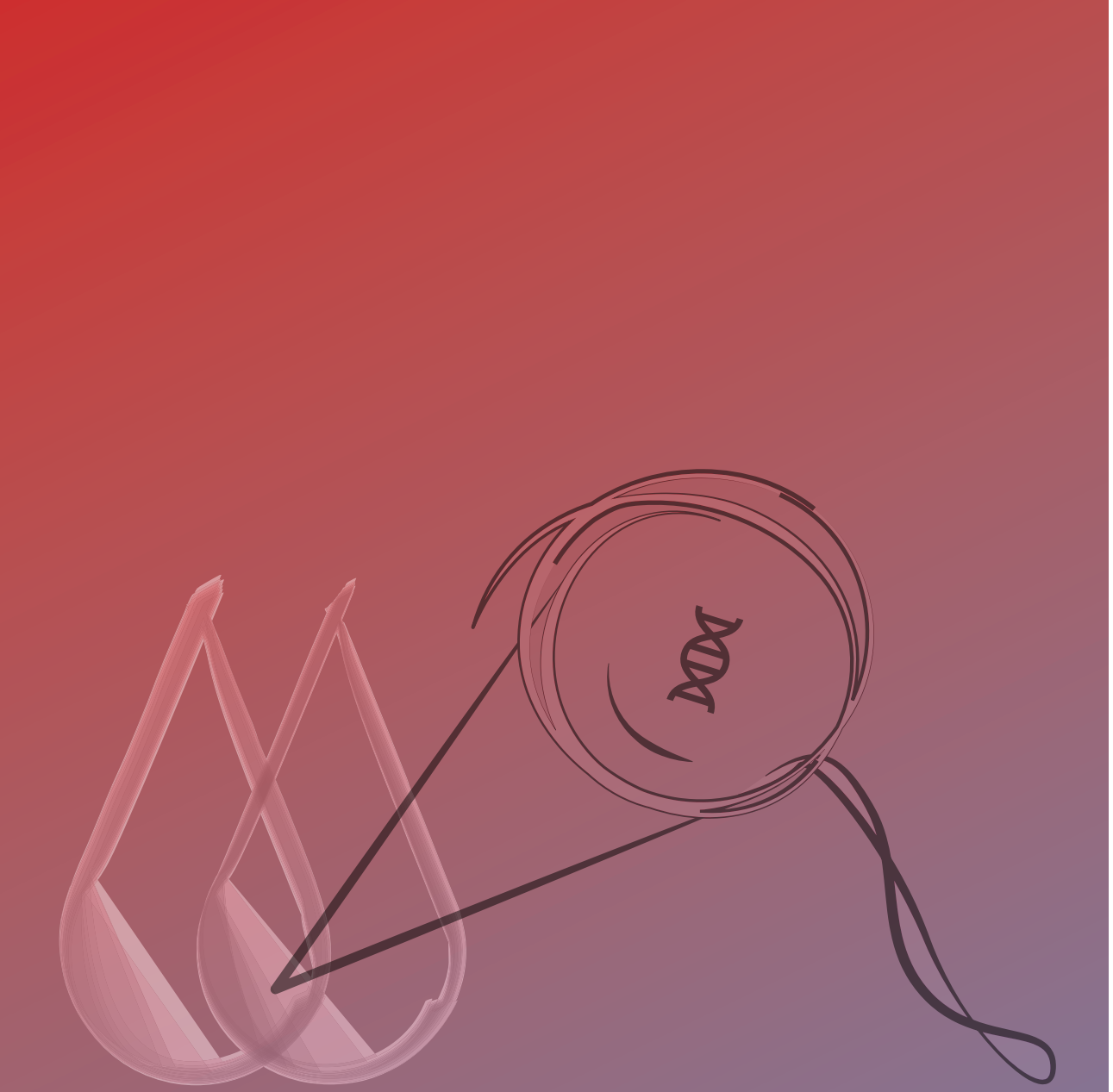


FIGURE S4. NTC samples showing minimal environmental contamination with W-positive droplets. No MT-positive droplets were detected in any of the NTC samples.



5

Pre-analytical blood sample workup for cell free DNA analysis using Droplet Digital PCR for future molecular cancer diagnostics

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ABSTRACT

In current molecular cancer diagnostics, using blood samples of cancer patients for the detection of genetic alterations in plasma (cell-free) circulating tumor DNA (ctDNA) is an emerging practice. Since ctDNA levels in blood are low, highly sensitive Droplet Digital PCR (ddPCR) can be used for detecting rare mutational targets. In order to perform ddPCR on blood samples, a standardized procedure for processing and analyzing blood samples is necessary to facilitate implementation into clinical practice. Therefore, we assessed the technical sample work-up procedure for ddPCR on blood plasma samples. Blood samples from healthy individuals, as well as lung cancer patients were analyzed. We compared different methods and protocols for sample collection, storage, centrifugation, isolation, and quantification. Cell-free DNA (cfDNA) concentrations of several wild type targets and *BRAF* and *EGFR*-mutant ctDNA concentrations quantified by ddPCR were primary outcome measurements. Highest cfDNA concentrations were measured in blood collected in serum tubes. No significant differences in cfDNA concentrations were detected between various time points of up to 24 hours until centrifugation. Highest cfDNA concentrations were detected after DNA isolation with the Quick-cfDNA Serum & Plasma Kit, while plasma isolation using the QIAamp Circulating Nucleic Acid Kit yielded the most consistent results. DdPCR results on cfDNA are highly dependent on multiple factors during pre-analytical sample workup, which need to be addressed during the development of this diagnostic tool for cancer diagnostics in the future.

INTRODUCTION

Current cancer diagnostics is often performed on molecular pathology findings from biopsy material. This is an invasive technique and not always possible to perform. A less invasive method for molecular diagnostics is the use of blood (i.e. 'liquid biopsy'). Blood samples are easy to obtain and contain cell-free DNA (cfDNA) including circulating tumor DNA (ctDNA). These DNA fragments carry patient-specific genetic targets and can be used as a diagnostic, prognostic, or predictive biomarker. This enables new strategies for personalized cancer medicine and other clinical fields like prenatal testing, transplantation medicine, and traumatology [1]. Highly sensitive droplet digital PCR (ddPCR) is capable of detecting these, often rare, targets [2, 3]. For optimal workflow, a standardized procedure is required for processing and cfDNA analysis [4, 5]. To date, different pre- and post-analytical approaches have been studied substantially for the development of molecular cancer diagnostics on liquid biopsies using quantitative PCR (qPCR) [6-9]. However, no standardized approach exists for the use of liquid biopsy in conjunction with ddPCR, which has only recently been introduced into molecular diagnostics and clinical research. This could explain inconsistencies in blood sample workup using ddPCR on liquid biopsies. We aim to generate a more standardized procedure for molecular testing on liquid biopsy using ddPCR. Here, we describe options of pre-analytical methods, in which we evaluated blood collection, storage of samples, centrifugation protocols, and isolation and quantification methods of cfDNA.

METHODS

Subjects and samples

Blood samples of 46 different healthy individuals and 18 lung cancer patients were used for analysis, as well as 3 pools each consisting of blood samples from 60 patients with various cancer types. Lung cancer patients were all NSCLC patients with progressive disease under erlotinib or gefitinib. Both primary driver mutation (*EFGR E746-A750del*, *G719S* or *L858R*) and resistance mutation (*T790M*) were analyzed during treatment. The healthy blood samples were retrieved from the anonymous blood donation biobank in the Utrecht University Medical Center (center A) and the Netherlands Cancer Institute (center B). Blood samples from cancer patients were all leftover material from the Netherlands Cancer Institute. According to Dutch national ethical guidelines, no ethical approval to use leftover material for scientific purposes is required, as the use of anonymous leftover material and clinical data for scientific purposes is part of the treatment agreement with patients [10].

Blood sample collection, room temperature storage and centrifugation

To test how blood sample collection, storage time, and centrifugation affects cfDNA quality and quantity, different materials and protocols were compared (Figure 1). All patient samples and methods used for analysis are summarized in Supplementary Table S1. Whole blood was collected in CellSave Preservative Tubes (Janssen Diagnostics, Raritan, NJ, USA), Cell-Free DNA BCT (Streck Inc, La Vista, NE, USA), K2/K3 ethylenediaminetetraacetic acid (EDTA), heparin, silicone coated (for serum separation) and citrate (BD Vacutainer, Franklin Lakes, NJ, USA) blood collection tubes (BCT).

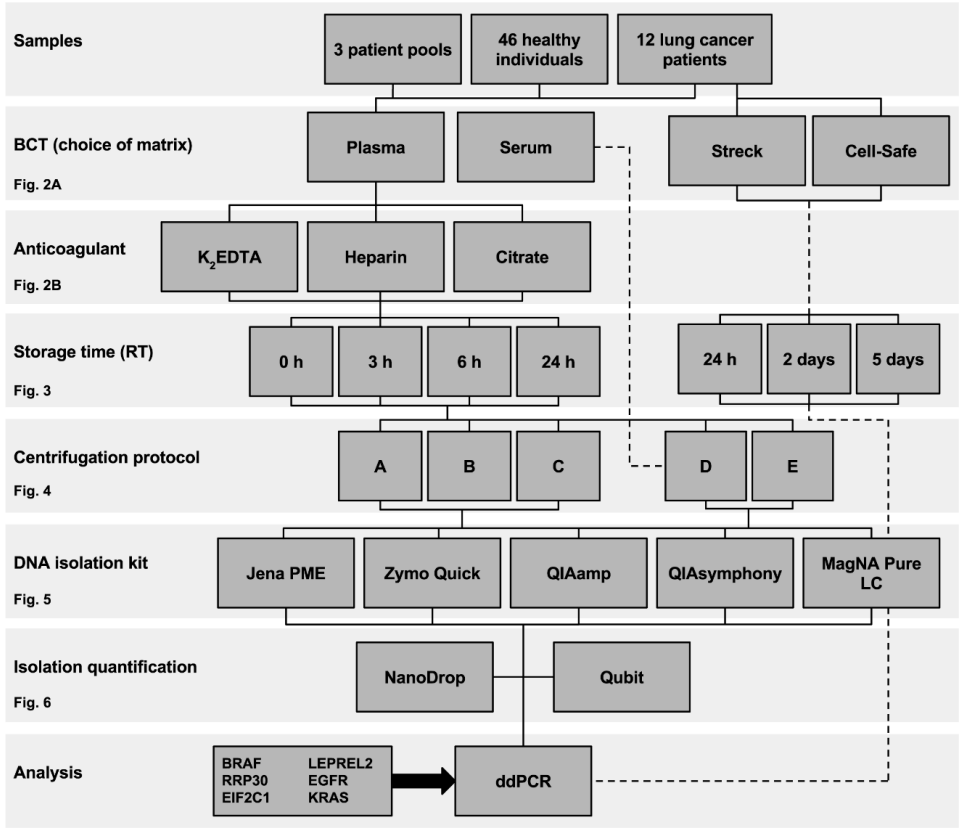

FIGURE 1. Summary of materials and methods used during various experiments. Please note this is a schematic overview of the experimental workflow. No exact experiments are depicted.

Samples were stored for 0 (T1), 3 (T2), 6 (T3), and 24 (T4) h at room temperature (RT) until centrifugation. Blood samples collected in Streck and CellSafe BCTs were stored for 24 (T4) h (P13-P14), 2 days (T5) (P15-P16), and 5 days (T6) (P17-P18). Centrifugation force could also affect levels of cfDNA as lysis of white blood cells in blood samples increase with increasing centrifugation force, which in turn increases background DNA concentration. Furthermore, less purified blood samples contain more cellular debris (e.g. proteins, nucleic acids), possibly leading to suboptimal DNA isolation and higher PCR interference. We performed and compared a one-step (slow-speed) and 4 different two-step (slow- and high-speed) centrifugation methods using the following centrifugation protocols of which parameters were based on standard clinical practice in both centers: (a) pre-freeze centrifugation for 10 minutes at 800g (Rotina 380, Hettich, Germany), (B) pre-freeze centrifugation for 10 minutes at 800g followed by microcentrifugation for 1 minute at 11,000g (5424 Microcentrifuge, Eppendorf, Germany), (C) pre-freeze centrifugation for 10 minutes at 800g pre-freeze followed by post-thaw microcentrifugation of 1 minute at 11,000g. Additionally, slightly modified protocols B (20 min at 380g and 10 min at 20,000g pre-freeze) and C (20 min at 380g pre-freeze and 10 min at 20,000g post- thaw) were handled, named protocol D and E respectively (Table 1). After each slow-speed centrifugation step, supernatant plasma was carefully removed from the tube and transferred into 1 ml aliquots. After each high-speed centrifugation step, supernatant plasma was transferred to a new 1.5 ml tube. All plasma samples were stored at -20°C.

DNA isolation methods

For comparison of DNA isolation methods the following isolation kits were used: Jena PME free circulating DNA extraction kit (Analytik Jena, Germany), QIAamp Circulating NA Kit (Qiagen, Hilden, Germany), QIAsymphony Circulating NA kit (Qiagen, Hilden, Germany), MagNAPure LC Total Nucleic Acid Isolation Large Volume kit (Roche Life Science, Basel, Switzerland), and Zymo Quick cfDNA serum & plasma kit (Zymo Research, Irvine, CA, USA). Kit specifications are shown in Table 2. All experiments were performed using 1 – 2 ml of plasma according to the manufacturer's protocol. Since the MagNA Pure kit allows a maximum plasma input of 1 ml, all other isolations methods were performed with 1ml plasma to gain comparable results. DNA isolated from healthy donor plasma was eluted in 50 µl elution buffer provided by the kit manufacturers. DNA isolated from plasma from lung cancer patients was eluted in 60 µl elution buffer.

TABLE 1. Centrifugation Protocols

Protocol	1st centrifugation			2nd centrifugation		
	Timing	Force (g)	Time (min)	Timing	Force (g)	Time (min)
A	Pre-freeze	800	10	Not performed	N/A	N/A
B	Pre-freeze	800	10	Pre-freeze	11,000	1
C	Pre-freeze	800	10	Post-thaw	11,000	1
D	Pre-freeze	380	20	Pre-freeze	20,000	10
E	Pre-freeze	380	20	Post-thaw	20,000	10

DNA isolation quantification

Quantification of nucleic acids within eluates after DNA isolation was performed using NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 3.0 fluorometry (Thermo Fisher Scientific) according to manufacturer's instructions (using 1 µl samples of DNA eluates). Both DNA quantification methods were analyzed by correlating measured values with ddPCR results of identical samples.

TABLE 2. Cell-free DNA isolation kit specifications

cfDNA isolation kit	Manufacturer	Plasma input (ml)	Technique	Carrier RNA
PME free-circulating DNA extraction kit	AnalytikJena	1 – 5	Spin-based	Optional
QIAamp Circulating NA Kit 50	Qiagen	3 – 5	Vacuum-based	Yes
QIAsymphony Circulating DNA Kit	Qiagen	4	Automated	Optional
MagNA Pure LC DNA Isolation Kit - Large Volume	Roche	1	Beads-assisted	No
Quick cfDNA serum & plasma kit	Zymo Research	10	Spin-based	No

ddPCR analysis

DdPCR was performed using several assays containing primers and probes targeting wild type *BRAF* (1), *RPP30* (2), *EIF2C1* (3), a single nucleotide polymorphism (SNP) variant of *LEPREL2* (4), 6 mutant *EGFR* (5-9), and 8 mutant *KRAS* (10 and 11 [G12/G13 Screening Multiplex Assay]). DNA templates used during PCR are shown in Supplementary Table S2 following MIQE guidelines for digital PCR [11]. Initial PCR mix volume consisted of 12 µl mastermix (11 µl Supermix for Probes [no uDTP] and 1 or 2 µl of wild type assay), and 9 or 10 µl of DNA depending on the amount of assay used. Within the no template control (NTC) DNA was substituted for purified H_2O (MilliQ, Billerica, MA, USA). All samples were analyzed in duplicate. PCR settings were based on a manually performed temperature gradient or validation data from Bio-Rad if available. Sample analysis of each experiment was performed using QuantaSoft v1.7.4.0917 software (Bio- Rad Laboratories, Hercules, CA, USA). Positive droplet concentrations in all samples were determined using manually placed fluorescence thresholds based on negative clusters as detected in the corresponding NTCs. Target DNA concentration (copies/µl) and absolute droplet counts within single samples were used as quantitative outcome measurement, while positive-to-total droplet ratios were calculated in order to compare efficiency of different isolation kits.

Statistical analysis

Paired differences in cfDNA yield were assessed by the Wilcoxon signed-rank test or

Friedman test with Dunn's correction in case of multiple intra-individual comparisons. Linear regression analysis was performed to calculate R^2 of DNA quantification measurement methods compared to ddPCR results. Statistical analysis was performed using GraphPad Prism software package version 6.02 (GraphPad Software, San Diego, CA, USA). Data are presented as medians with interquartile range (mdn, q1 – q3), or as means with standard deviation (mn±sd). For all comparisons, a value of $p < 0.05$ was considered to be significant (two-tailed).

RESULTS

Blood collection

PCR results of blood plasma and serum samples from 10 healthy blood donors (D1-D10) were compared using the MagNA Pure kit and another 15 healthy blood donors (D11-D25) were compared using the QIAamp kit isolation method. In all 25 cases, cfDNA concentrations were significantly highest in serum samples compared to paired EDTA samples (204.0 [67.7 – 532.0] vs. 18.4 [12.7 – 21.4], $p < 0.001$) (Figure 2A). In a second experiment, four different BCTs (EDTA, heparin, serum, citrate) were compared in 8 different healthy blood donors (D26-D33). In all cases the Zymo kit was used for cfDNA isolation between T1 and T2. Median cfDNA concentrations (copies/µl) were significantly higher in serum samples compared to paired citrate samples (206.0 [193.5 – 352.3] vs. 30.8 [24.2 – 46.4], $p < 0.05$) and heparin samples (206.0 [193.5 – 352.3] vs. 106.5 [15.7 – 205.8], $p < 0.05$). Furthermore, significantly higher cfDNA concentrations were found in EDTA samples compared to paired heparin samples (488.5 [28.5 – 966.3] vs. 106.5 [15.7 – 20.5], $p < 0.05$) (Figure 2B and Supplementary Figure S1).

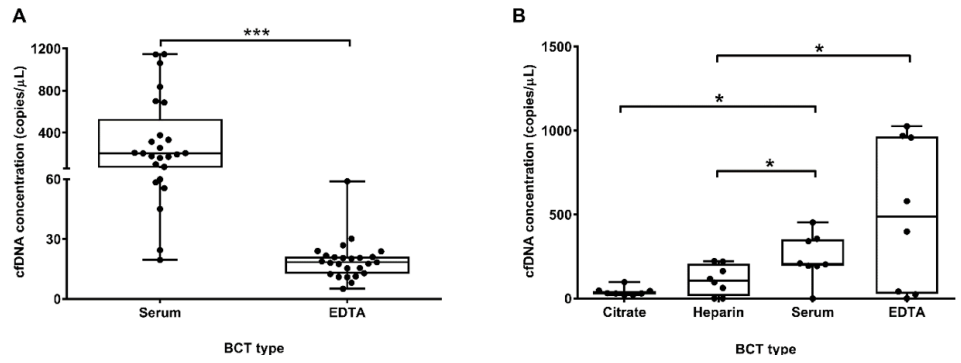

FIGURE 2. Comparison of cfDNA concentrations in paired blood samples in 4 different BCTs. All samples were collected from healthy controls. In all experiments assay 1 was used during ddPCR.

The boxplots indicate cfDNA concentrations on the y-axis, comparing serum with EDTA BCTs from 25 healthy controls on the x-axis (A), and citrate, heparin, serum, and EDTA BCTs from 8 other healthy controls (B). The crossing lines indicate medians, the upper and lower limits of the boxes indicate interquartile ranges (25^{th}/75^{th} percentiles), and whiskers represent minima and maxima. *P < 0.05, **P < 0.01, ***P < 0.001.

Blood storage time until centrifugation

Average total cfDNA concentrations of the 3 blood sample pools were evaluated for storage time until centrifugation at consecutive time points T1-T4. DNA was isolated using MagNA Pure and QIAsymphony kits. Medians of pooled averages ranged from 74.4 – 84.1 copies/µl using QIAsymphony, compared to 147.8 – 177.1 copies/µl using MagNA Pure (Figure 3A). Additionally, EDTA samples from six individual subjects (D34-D39) were stored at RT and centrifuged following protocol A at consecutive time points T1-T4. DNA was isolated using the Zymo Quick kit. Median cfDNA concentrations did not show any significant differences (p = 0.910) between time points T1-T4 using paired analysis (Figure 3B and Supplementary Figure S2). We also tested cfDNA stability in Streck and CellSave BCTs by comparing mean mutant fractions of cfDNA concentrations in blood samples from 2 lung cancer patients per time point (T4: P13-P14; T5: P15-P16; T6: P17-P18) after centrifugation using protocol D (Figure 3C). For directly isolated EDTA samples (n = 6), the mutant fraction of mean total cfDNA concentration was 16.0%, compared to mutant fractions of 18.0% and 14.8% at T4 (n=2), 2.1% and 2.6% at T5 (n=2), and 22.5% and 22.3% at T6 (n=2) in Streck and CellSave samples, respectively.

Centrifugation protocol

Centrifugation protocols A, B, and C were performed at T2 (3 h) and compared using EDTA plasma from healthy individuals D37-D42, while modified centrifugation protocols D and E were performed on patient samples P1-P5. The median cfDNA concentration detected after centrifugation using protocol A was (72.0 [22.3 – 156.5]) copies/µl, compared to (27.7 [15.0 – 42.3]) copies/µl using protocol B and (36.2 [15.3 – 122.6]) copies/µl using protocol C. The median cfDNA concentration detected after using protocol D was (31.5 [19.2 – 65.0]) copies/µl, compared to (39.8 [26.4 – 68.5])] copies/µl for protocol E (Figure 4 and Supplementary Figure S3).

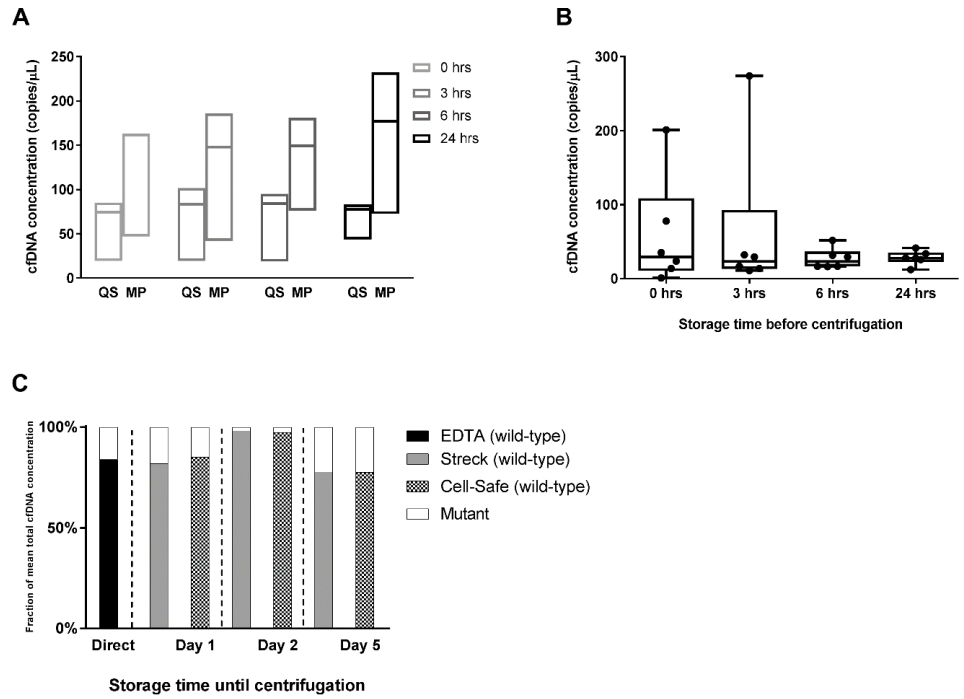

FIGURE 3. Influence of storage time on cfDNA concentrations until centrifugation. Time points T1-T6 are depicted on the x-axes. Median cfDNA concentrations were depicted on the y-axes for average yields of pooled EDTA samples after analysis using assay 1 (A), and paired EDTA samples from 6 healthy individuals after using assay 2 (B). In 6 blood samples collected in Streck and CellSave BCTs using assay 6,7,10-12 (C). At each consecutive time point, mean mutant and wild-type cfDNA concentrations from samples of 2 other individuals were compared with the mean cfDNA concentration of the matching EDTA samples, as depicted by mutant/wild-type fractions (y-axis). QS QIAsymphony, MP MagnaPure.

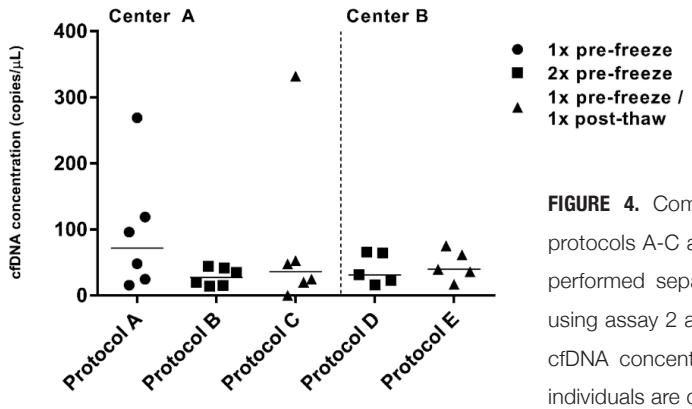

FIGURE 4. Comparisons of centrifugation protocols A-C and D-E. Comparisons were performed separately in centers A and B using assay 2 and 5, respectively. Absolute cfDNA concentrations (y-axis) detected in individuals are depicted for each protocol.

Isolation method

Isolation kits were tested on EDTA samples from D43-D46, using centrifugation protocol A at T2 (3 h). Droplet read-out of samples (n=4) revealed a mean amount of positive droplets of 2,875±1,864 and total droplets of 15,261±1,196 with Jena PME, a mean positive droplets of 5,086±2,966 and total droplets of 11,869±861 with QIAamp, and a mean positive droplets of 7,339±4,867 and mean total droplets of 13,511±1,460 with Zymo Quick kit (Figure 5A/B).This resulted in positive-to-total droplets ratios of 0.19, 0.43, and 0.54, respectively. Identical findings were detected using assay 4 (Supplementary Figure S4). In patient samples P6-P12, centrifuged using protocol B at T2, the median total positive droplets detected after DNA isolation with the QIAamp kit was significantly higher compared to that detected after isolation with the MagNA Pure kit (316 [199 – 521] vs. 213 [162 – 344], $p < 0.05$). No significant differences were found between median positive droplets by QIAsymphony (236 [132 – 489]) and the other two kits. Mutant positive droplets were only detected in P9, 11, and 12, but did not differ significantly (Figure 5C). The QIAsymphony kit significantly yielded the highest amount of total droplets compared to both QIAamp (14,942 [13,825 – 16,246] vs. 13,645 [12,752 – 14,132], $p < 0.05$) and MagNA Pure (14,942 [13,825 – 16,246] vs. 13,705 [12,260 – 15,028], $p < 0.05$) kits. No significant differences were found between QIAamp and MagNA Pure kits (Figure 5D).

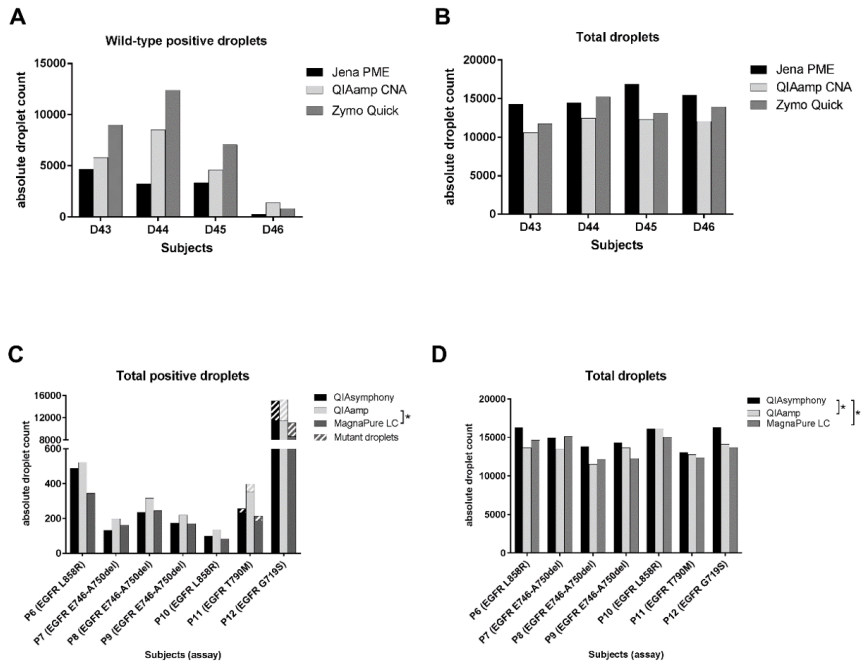

FIGURE 5. Isolation methods in healthy individuals and cancer patients. Absolute droplets counts are shown on the y-axes. For healthy individuals, wild-type (A) and total positive droplet (B) yield using assay 1 were depicted. For cancer patients, the sum of mutant and wild-type positive droplets (C), as well as total droplet yields (D) where depicted using assay 6-9. *P < 0.05.

DNA quantification prior to ddPCR

In order to assess accuracy of DNA quantification methods, results of NanoDrop and Qubit were compared to ddPCR results of identical plasma samples from healthy individuals. DNA quantification of samples using NanoDrop resulted in a non-significant R^2 for ddPCR using assay 3 (Figure 6A), whereas Qubit results yielded R^2 of 0.96 (β = 1.06 [CI 1.01 - 1.10]), $p < 0.0001$) for ddPCR (Figure 6B). Results using assay 1 during ddPCR are shown in Supplementary Figure S5.

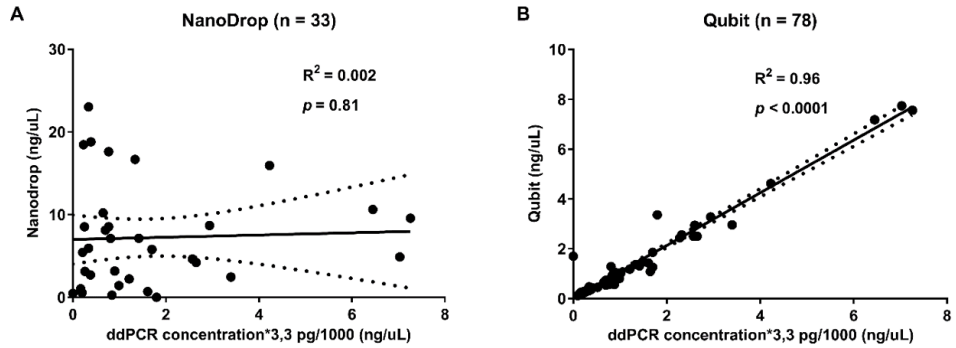

FIGURE 6. DNA quantification after isolation of EDTA samples using assay 3. In order to perform linear regression, all ddPCR results were adhered to NanoDrop and Qubit quantification results assuming 3.3 pg DNA/haploid genome (x-axes) and depicted as ng/µl (y-axis). In total, 38 samples were quantified by NanoDrop (A), of which 5 results were negative values and excluded from analysis. Seventy-eight samples were quantified using Qubit (B). R^2 represents goodness-of-fit of DNA quantification methods for ddPCR.

DISCUSSION

This study shows that ddPCR results of cfDNA quantification strongly depend on pre-analytical blood sample workup, comprising an extensive multi-step process. Our series of paired serum and plasma samples show a significant increase in median cfDNA concentrations of roughly 10 times in serum samples, which is consistent with several studies of comparable size reporting 2 – 24 times higher cfDNA concentrations detected

by quantitative PCR in serum compared to plasma samples [12-15]. In our comparison of ddPCR results in various BCTs, no significant differences were found between ddPCR results of paired plasma and serum samples. This might have been the result of differences in sample size or workup. Furthermore, a significant increase in cfDNA concentrations has been previously observed in serum compared to plasma samples after storage time at room temperature and 4°C for up to 24 h [13]. Possible explanations given for the increased DNA detection in serum samples are extracorporeal release of cfDNA from white blood cells (WBC) in serum through WBC lysis during whole blood transportation and centrifugation, or by stored clotted blood [16].

We assessed cfDNA concentrations in blood samples at different time points after isolation using 2 different methods. Our pooled data show that cfDNA concentrations can remain stable in EDTA plasma over 24 h until centrifugation. Furthermore, we found significantly stable cfDNA concentrations intra-individually, supported by ddPCR results of paired samples obtained at different time points. However, firm conclusions cannot be drawn due to small samples sizes. In one case, we observed an unexplained spike in cfDNA concentration at 3 h after ddPCR performed with two different assays. Previous studies showed no differences in cfDNA levels in plasma samples centrifuged at different time points up to 24 h [13, 16]. On the contrary, several other studies reported significant increases in plasma cfDNA levels after storage of EDTA plasma up to 72 h at both room temperature and at 4°C to various degrees [6, 14, 17, 18]. Differences in cfDNA yields among various studies might be the result of differences in sample handling and/or storage temperature, as this might affect WBC stability accompanied by the possible release of genomic DNA similar to serum samples [19].

Regarding cancer diagnostics, the detection of cfDNA mostly involves detecting mutations in ctDNA, which are often (rare) targets of interest within a background of wild type cfDNA [6]. Therefore, variation in background DNA concentration during workup of serum samples is unwanted and should be avoided using plasma samples instead [20, 21]. Furthermore, whole blood samples can be collected and stored for plasma analysis in BCTs containing different kinds of anticoagulants with potential ddPCR inhibiting features. Our results show that citrate and heparin anticoagulants yielded the smallest amounts of cfDNA from the same samples compared to serum and EDTA samples. This corresponds with previous qPCR results on different BCT types, where EDTA was preferred as anticoagulant above citrate or heparin as cfDNA concentrations appear to be more stable over time within EDTA matrix compared to citrate or heparin [22, 23]. Palmirotta et al. compared DNA quality and quantity in blood plasma from healthy donors collected in 6 different BCTs containing different anticoagulants. Highest DNA purity and concentrations were reported for samples originating from citrate and EDTA plasma BCTs compared to heparin and fluoride-oxalate BCTs, as measured by spectrophotometry, gel-electrophoresis and qPCR [24]. More recently, newer commercially available BCTs specifically designed for cfDNA analysis are Cell-Free DNA™ by Streck and CellSave by Janssen Diagnostics, which show even more stable plasma cfDNA concentrations after storage for 48 h up to 14 days compared to K2/K3-EDTA BCTs [18, 25-30]. Therefore, the use of cfDNA and CellSave BCTs could be particularly beneficial for plasma cfDNA testing in large multicenter studies, in which storage time until centrifugation can practically be >24 h.

The centrifugation protocol for plasma collection also affects cfDNA concentration. Blood cells first have to be removed by slow centrifugation in order to avoid cell lysis and unwanted release of genomic DNA, whereas cellular remnants will be removed afterwords by short-term high-speed microcentrifugation, either before or after a freeze-thaw cycle [31]. We observed a 2.5–3.0-fold decrease in plasma cfDNA concentrations after a two-step centrifugation compared to a single-step slow-speed centrifugation, which corresponds with previous data on protocols using similar centrifugation parameters and qPCR to quantify results [32, 33]. We detected comparable results between two-step protocols with modified centrifugation forces and time parameters showing no significant differences in cfDNA yield, which is consistent with previous data on high-speed centrifugation [33, 34]. These results proof the (potentially unwanted) release of genomic DNA into the sample by remaining cellular material, and emphasizes the need for a second plasma filtering step by microcentrifugation, either pre- or post-thaw, in order to prevent contamination with cellular DNA and retrieve purely plasma cfDNA. Data on plasma storage conditions after centrifugation (e.g. time, temperature, freeze-thaw cycles) are scarce. Previously performed qPCR experiments showed comparable cfDNA yields for different parameters [6, 20]. Furthermore, evidence exists that repeated freeze-thaw cycles of stored plasma samples prior to DNA isolation leads to cfDNA fragmentation [6, 8, 14].

Plasma DNA isolation can be performed using different methods supplied by a vast amount of manufacturers. Although sample sizes were small, we show that the 5 different DNA isolation kits we assessed performed all reasonably well; Zymo Quick kit seemed to perform most efficiently in combination with ddPCR compared to the QIAamp and Jena PME kits, as highest absolute and relative concentrations of cfDNA were detected in plasma samples. On the other hand, the QIAamp kit showed the lowest coefficient of variation for both positive and total droplet yields, suggesting this kit to perform most consistently compared to the other two kits. Especially concerning cancer diagnostics (e.g. treatment response monitoring by serial quantification of ctDNA), consistency is an important factor to consider as only consistent quantification results would allow for reliable evaluation of tumor dynamics. Besides, the QIAamp kit revealed significantly higher droplet yields compared to the MagNA Pure kit. QIAamp and QIAsymphony performed equally well with regard to positive droplet yields. In a similar comparison with Jena PME and QIAsymphony, the QIAamp kit yielded highest concentrations of mutant KRAS in plasma samples from non-small cell lung cancer patients [18]. In two other studies, Norgen Plasma/Serum Circulating

DNA Purification Mini Kit yielded slightly better isolation results compared to QIAamp kit depending on cancer type and used assay [9], while ctDNA in plasma samples of early stage (*KRAS*-mutated) pancreatic cancer patients was not being detected using ddPCR after isolation with QIAamp kit [35]. Thus, isolation results not only vary strongly across different DNA isolation kits, but also between experiments performed with the same kit. Therefore, results not only depend on the used kit itself, but also on the circumstances and parameters used during experimental workup such as patient characteristics, tumor type and stage, target type, DNA input volume, and analysis technique. Overall, we experienced best ddPCR results of DNA isolation kits using Zymo Quick and QIAamp kits.

DNA isolation quantity could be checked by fluorospectroscopy or fluorometry of DNA eluates. R^2 demonstrated a poor predictive ability of NanoDrop quantification measurements in respect to ddPCR cfDNA concentrations of eluted DNA samples, while a strong significant correlation was found between Qubit quantification measurements and ddPCR results of these samples. Similar results were previously shown for experiments using qPCR [9]. We observed no correlation between NanoDrop measurements and ddPCR results. Therefore, we recommend a DNA isolation quantification check using Qubit fluorometry before proceeding to actual PCR analysis. This could enhance efficiency during workflow by avoiding wasting of time and costly materials used during ddPCR, in case of insufficient DNA isolated.

Several variables during preparation of actual ddPCR can affect analysis results. For instance, the amount of DNA sample input to be analyzed, which largely depends on the purpose of testing; maximum available amounts of DNA sample are desired in case of rare target detection (e.g. cancer diagnostics, post-transplantation monitoring), while for copy number variation analysis (e.g. prenatal diagnostics) the amount of required DNA depends on the expected highest target copy number. Assays used for ddPCR need to be validated separately, because fluorescence values used for readout of positive and negative droplets can vary depending on PCR inhibitors present in DNA matrix or assay design. First, optimal ddPCR settings (i.e. ramp-rate and annealing time) should be determined by temperature gradients performed on positive control samples with similar DNA matrix. Subsequently, the limit of detection (LOD) needs to be determined by estimating false-positive rate through running strings of wild-type-only control and NTC samples [36]. During post-PCR analysis, technical errors such as reduced or increased fluorescent signals from damaged positive droplets or negative droplets can cause droplets to be displayed in between positive or negative clusters, which is defined as ‘rain’. This hampers accurate post-PCR analysis. Third parties already designed methods to improve automated thresholding by either *k*-nearest neighbor clustering, ‘extreme value methodology’, or kernel density estimation with Gaussian mixture models [37-39]. Lastly, the interpretation of results remains subject of discussion. Positive targets in Quantasoft are being reported as copies/µl reaction

volume, which is calculated using the number of droplets detected and droplet volume (nano sized) [40]. Subsequently, this could manually be converted to copies/ml plasma and whole blood using input volume of DNA sample for ddPCR and plasma volume for DNA isolation. However, large conversion factors between these volumes could easily render errors in estimating target copy concentrations in blood. Therefore, standardization of input volumes for analysis is pivotal. This further raises important questions about the clinical relevance of the acquired results; what is the significance of a decrease or increase of target concentrations in clinical management? At what target concentration should (targeted) therapy be started and/or adjusted?

In conclusion, we recommend a two-step centrifugation protocol for separating plasma collected in EDTA BCTs for storage until cfDNA isolation within 24 h. Furthermore, the Zymo Quick kit yielded best results quantitatively for cfDNA isolation compared to others. The QIAamp kit seems to be most consistent and yielded highest cfDNA concentrations compared to the QIAsymphony and MagNA Pure kits. Furthermore, we think that Qubit fluorometry for a quantity check of cfDNA isolation might enhance workflow efficiency towards ddPCR analysis. And, although further clinical research and technical refinements of ddPCR analysis are needed for incorporation into clinical practice, improving overall efficiency in sample workup is an inevitable first step.

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AUTHOR CONTRIBUTIONS

JH, DA, and MMH developed concepts, designed the experiments, validated reproducibility of the experiments/results, and analyzed the data. J and D performed the experiments. JH, DA, R, SM, and MMH wrote the article. JH performed statistical analysis and visualization of the data. DA, J, D, R, and MMH provided study materials and/or instrumentation. DA, SM and MMH supervised JH. DA and SM financially supported the study project.

REFERENCES

1. Lui YYN, Lo YMD. Circulating DNA in plasma and serum: Biology, preanalytical issues and diagnostic applications. Clin Chem Lab Med. 2002;40:962-8.
2. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical chemistry. 2011;83:8604-10.
3. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods. 2013;10:1003-5.
4. Mazaika E, Homsy J. Digital Droplet PCR: CNV Analysis and Other Applications. Curr Protoc Hum Genet. 2014;82:7 24 1-13.
5. Vossen RH, White SJ. Quantitative DNA Analysis Using Droplet Digital PCR. Methods Mol Biol. 2017;1492:167-77.
6. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: Preanalytical considerations. Clinica chimica acta; international journal of clinical chemistry. 2013;424:222-30.
7. Elshimali YI, Khaddour H, Sarkissyan M, Wu Y, Vadgama JV. The clinical utilization of circulating cell free DNA (CCFDNA) in blood of cancer patients. International journal of molecular sciences. 2013;14:18925-58.
8. Bronkhorst AJ, Aucamp J, Pretorius PJ. Cell-free DNA: Preanalytical variables. Clinica chimica acta; international journal of clinical chemistry. 2015;450:243-53.
9. Mauger F, Dulary C, Daviaud C, Deleuze JF, Tost J. Comprehensive evaluation of methods to isolate, quantify, and characterize circulating cell-free DNA from small volumes of plasma. Analytical and bioanalytical chemistry. 2015;407:6873-8.
10. van Diest PJ. For and against - No consent should be needed for using leftover body material for scientific purposes - For. Brit Med J. 2002;325:648-9.
11. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, et al. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin Chem. 2013;59:892-902.
12. Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. Transfusion. 2001;41:276-82.
13. Jung M, Klotzek S, Lewandowski M, Fleischhacker M, Jung K. Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem. 2003;49:1028-9.
14. Chan KC, Yeung SW, Lui WB, Rainer TH, Lo YM. Effects of preanalytical factors on the molecular size of cell-free DNA in blood. Clin Chem. 2005;51:781-4.
15. Umetani N, Hiramatsu S, Hoon DSB. Higher amount of free circulating DNA in serum than in plasma is not mainly caused by contaminated extraneous DNA during separation. Ann Ny Acad Sci. 2006;1075:299-307.
16. Board RE, Williams VS, Knight L, Shaw J, Greystoke A, Ranson M, et al. Isolation and extraction of circulating tumor DNA from patients with small cell lung cancer. Ann N Y Acad Sci. 2008;1137:98-107.
17. Xue X, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. Clinica chimica acta; international journal of clinical chemistry. 2009;404:100-4.
18. Sherwood JL, Corcoran C, Brown H, Sharpe AD, Musilova M, Kohlmann A. Optimised Pre-Analytical Methods Improve KRAS Mutation Detection in Circulating Tumour DNA (ctDNA) from Patients with Non-Small Cell Lung Cancer (NSCLC). PloS one. 2016;11:e0150197.
19. Tatsumi N, Miwa S, Lewis SM, International Society of H, the Intrnational Council for Standardization in H. Specimen collection, storage, and transmission to the laboratory for hematological tests. Int J Hematol. 2002;75:261-8.
20. Sozzi G, Roz L, Conte D, Mariani L, Andriani F, Verderio P, et al. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. Journal of the National Cancer Institute. 2005;97:1848-50.
21. Page K, Powles T, Slade MJ, MT DEB, Walker RA, Coombes RC, et al. The importance of careful blood processing in isolation of cell-free DNA. Ann N Y Acad Sci. 2006;1075:313-7.
22. Lam NYL, Rainer TH, Chiu RWK, Lo YMD. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. Clin Chem. 2004;50:256-7.
23. Permenter J, Ishwar A, Rounsavall A, Smith M, Faske J, Sailey CJ, et al. Quantitative analysis of genomic DNA degradation in whole blood under various storage conditions for molecular diagnostic testing. Mol Cell Probe. 2015;29:449-53.
24. Palmirotta R, Ludovici G, De Marchis ML, Savonarola A, Leone B, Spila A, et al. Preanalytical Procedures for DNA Studies: The Experience of the Interinstitutional Multidisciplinary BioBank (BioBIM). Biopreserv Biobank. 2011;9:35-45.
25. Norton SE, Luna KK, Lechner JM, Qin JB, Fernando MR. A New Blood Collection Device Minimizes Cellular DNA Release During Sample Storage and Shipping When Compared to a Standard Device. J Clin Lab Anal. 2013;27:305-11.
26. Norton SE, Lechner JM, Williams T, Fernando MR. A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR. Clinical biochemistry. 2013;46:1561-5.
27. Kang Q, Henry NL, Paoletti C, Jiang H, Vats P, Chinnaiyan AM, et al. Comparative analysis of circulating tumor DNA stability In K3EDTA, Streck, and CellSave blood collection tubes. Clinical biochemistry. 2016.
28. Parpart-Li S, Bartlett B, Popoli M, Adleff V, Tucker L, Steinberg R, et al. The effect of preservative and temperature on the analysis of circulating tumor DNA. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016.
29. Rothwell DG, Smith N, Morris D, Leong HS, Li YY, Hollebecque A, et al. Genetic profiling of tumours using both circulating free DNA and circulating tumour cells isolated from the same preserved whole blood sample. Molecular oncology. 2016;10:566-74.
30. Medina Diaz I, Nocon A, Mehnert DH, Fredebohm J, Diehl F, Holtrup F. Performance of Streck cfDNA Blood Collection Tubes for Liquid Biopsy Testing. PloS one. 2016;11:e0166354.
31. Gormally E, Hainaut P, Caboux E, Airoldi L, Autrup H, Malaveille C, et al. Amount of DNA in plasma and cancer risk: a prospective study. International journal of cancer Journal international du cancer. 2004;111:746-9.
32. Chiu RW, Poon LL, Lau TK, Leung TN, Wong EM, Lo YM. Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. Clin Chem. 2001;47:1607-13.
33. Swinkels DW, Wiegerinck E, Steegers EA, de Kok JB. Effects of blood-processing protocols on cell-free DNA

quantification in plasma. Clin Chem. 2003;49:525-6.

34. Kadam SK, Farmen M, Brandt JT. Quantitative measurement of cell-free plasma DNA and applications for detecting tumor genetic variation and promoter methylation in a clinical setting. The Journal of molecular diagnostics : JMD. 2012;14:346-56.
35. Sorber L, Zwaenepoel K, Deschoolmeester V, Roeyen G, Lardon F, Rolfo C, et al. A Comparison of Cell-Free DNA Isolation Kits: Isolation and Quantification of Cell-Free DNA in Plasma. The Journal of molecular diagnostics : JMD. 2016.
36. Castellanos-Rizaldos E, Paweletz C, Song C, Oxnard GR, Mamon H, Janne PA, et al. Enhanced ratio of signals enables digital mutation scanning for rare allele detection. The Journal of molecular diagnostics : JMD. 2015;17:284-92.
37. Jones M, Williams J, Gartner K, Phillips R, Hurst J, Frater J. Low copy target detection by Droplet Digital PCR through application of a novel open access bioinformatic pipeline, 'definetherain'. J Virol Methods. 2014;202:46-53.
38. Trypsteen W, Vynck M, De Neve J, Bonczkowski P, Kiselinova M, Malatinkova E, et al. ddpcRquant: threshold determination for single channel droplet digital PCR experiments. Analytical and bioanalytical chemistry. 2015;407:5827-34.
39. Attali D, Bidshahri R, Haynes C, Bryan J. ddpcr: an R package and web application for analysis of droplet digital PCR data. F1000Res. 2016;5:1411.
40. Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, et al. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Analytical chemistry. 2012;84:1003-11.

SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE S1.

				Applied method		
Subject	**Patient type**	**BCT[1]**	**Storage time (T)[2]**	**Centrifugation[3]**	**DNA isolation[4]**	**Assay[5]**
D1	Healthy donor	E, S	1	D	M	1
D2	Healthy donor	E, S	1	D	M	1
D3	Healthy donor	E, S	1	D	M	1
D4	Healthy donor	E, S	1	D	M	1
D5	Healthy donor	E, S	1	D	M	1
D6	Healthy donor	E, S	1	D	M	1
D7	Healthy donor	E, S	1	D	M	1
D8	Healthy donor	E, S	1	D	M	1
D9	Healthy donor	E, S	1	D	M	1
D10	Healthy donor	E, S	1	D	M	1
D11	Healthy donor	E, S	1	D	QA	1
D12	Healthy donor	E, S	1	D	QA	1
D13	Healthy donor	E, S	1	D	QA	1
D14	Healthy donor	E, S	1	D	QA	1
D15	Healthy donor	E, S	1	D	QA	1
D16	Healthy donor	E, S	1	D	QA	1
D17	Healthy donor	E, S	1	D	QA	1
D18	Healthy donor	E, S	1	D	QA	1
D19	Healthy donor	E, S	1	D	QA	1
D20	Healthy donor	E, S	1	D	QA	1
D21	Healthy donor	E, S	1	D	QA	1
D22	Healthy donor	E, S	1	D	QA	1
D23	Healthy donor	E, S	1	D	QA	1
D24	Healthy donor	E, S	1	D	QA	1
D25	Healthy donor	E, S	1	D	QA	1
D26	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D27	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D28	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D29	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D30	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D31	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D32	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D33	Healthy donor	E, H, S, C	1	A	Z	1,2,3
D34	Healthy donor	E	1,2,3,4	A	Z	2,3
D35	Healthy donor	E	1,2,3,4	A	Z	2,3

SUPPLEMENTARY TABLE 1 CONTINUED.

			Applied method				
Subject	**Patient type**	**BCT[1]**	**Storage time (T)[2]**	**Centrifugation[3]**	**DNA isolation[4]**	**Assay[5]**
D36	Healthy donor	E	1,2,3,4	A	Z	2,3
D37	Healthy donor	E	1,2,3,4	A,B,C	Z	2,3
D38	Healthy donor	E	1,2,3,4	A,B,C	Z	2,3
D39	Healthy donor	E	1,2,3,4	A,B,C	Z	2,3
D40	Healthy donor	E	2	A,B,C	Z	2,3
D41	Healthy donor	E	2	A,B,C	Z	2,3
D42	Healthy donor	E	2	A,B,C	Z	2,3
D43	Healthy donor	E	2	A	J, Z, QA	1,4
D44	Healthy donor	E	2	A	J, Z, QA	1,4
D45	Healthy donor	E	2	A	J, Z, QA	1,4
D46	Healthy donor	E	2	A	J, Z, QA	1,4
P1	NSCLC	E, S	2	D, E	QS	1
P2	NSCLC	E, S	2	D, E	QS	1
P3	NSCLC	E, S	2	D, E	QS	1
P4	NSCLC	E, S	2	D, E	QS	1
P5	NSCLC	E, S	2	D, E	QS	1
P6	NSCLC	E	2	D	M, QA, QS	5
P7	NSCLC	E	2	D	M, QA, QS	6
P8	NSCLC	E	2	D	M, QA, QS	6
P9	NSCLC	E	2	D	M, QA, QS	5
P10	NSCLC	E	2	D	M, QA, QS	7
P11	NSCLC	E	2	D	M, QA, QS	8
P12	NSCLC	E	2	D	M, QA, QS	6
P13	NSCLC	E, St, CS	1,4	D	M	9
P14	NSCLC	E, St, CS	1,4	D	M	11
P15	NSCLC	E, St, CS	1,5	D	M	10
P16	NSCLC	E, St, CS	1,5	D	M	5
P17	NSCLC	E, St, CS	1,6	D	M	6
P18	NSCLC	E, St, CS	1,6	D	M	11
Pool 1	Various cancers	E	1,2,3,4	D	M, QS	1
Pool 2	Various cancers	E	1,2,3,4	D	M, QS	1
Pool 3	Various cancers	E	1,2,3,4	D	M, QS	1

1. E EDTA; S, Serum; H, Heparin; C, Citrate; St, Streck BCT; CS, CellSave BCT.
2. 1 = direct; 2 = 3 h; 3 = 6 h; 4 = 24 h; 5 = 48 h; 6 = 5 days.
3. A, 1x pre-freeze; B, 2x pre-freeze; C, 1x pre-freeze/1x post-thaw; D, 2x pre-freeze; E, 1x pre-freeze/1x post-thaw.
4. J, Jena PME; QA, QIAamp; QS, QIAsymphony; M, MagNA Pure; Z, Zymo Quick.
5. 1, $BRAF_{intron}$; 2, $RRP30_{intron}$; 3, $EIF2C1_{intron}$; 4, LEPREL2intron; 5, $EGFR_{c.2236G>C}$; 6, $EGFR_{c.2235\_2249del15}$; 7, $EGFR_{c.2369C>T}$; 8, $EGFR_{c.2156G>A}$; 9, $EGFR_{c.2582T>A}$; 10, $KRAS_{c.34G>T}$; 11, $KRAS_{multiplex}$

SUPPLEMENTARY TABLE S2.1. Overview of ddPCR assays following MIQE guidelines

Assay	Supplier	ID	Gene	Chromo-some mapping	Chromosome location	variant	Protein change	Amplicon Context [FAM/HEX]	Amplicon length
1	Bio-Rad	dHsa CP20 00028	BRAF	7q34	7:140500162-40500284	Intron	N/A	CACTCCATCGAGATTTCACTGTAGCTAGACCA AAATCACCTA TTTTTACTGTGAGGTCTTCATGA AGAAATATATCTGAGGTGTA GTAAGTAAAGGA AAACAGTAGATCTCATTTTCCTATCA	91
2	Bio-Rad	dHsa CP10 00485	RRP30	10q23.31	10:92634445-92634567	Intron	N/A	TTAAGTAACTTGTAAGTGGTAGTGCATAGACT TTAAATCAGGC AGACTGACACTAGAGTTCACA TTCATAACCACTCCTCAAATG TCCTCCTACTCT TGACATCTAGACTCAGGATGGACCTG	98
3	Bio-Rad	dHsa CP10 00484	EIF2C1	1p34.3	1:36359357-36359479	Intron	N/A	GGCTTTCACCAGTCTGTGCGCCCTGCCATGT GGAAGATGAT GCTCAACATTGATGGTGAGTGGGGAGAGCTATGGAGCCAGG GGCACCCCAA GTCCAGTGACCACACTCCCAGCCTCATCC CT	86
4	Life techno-logies	rs226 9355	LEPREL2	12q13	12:6836700-6836800	Intron	N/A	TGTAGGAAGCTCTCCCGAGTTCTCT[G/C]CACAGTCCCCTT AGTAAGCGGGATT	51
5	Bio-Rad	dHsa CP20 00021	EGFR	7p12	7:55259483-55259605	c.2236G>C	L858R	ACCGCAGCATGTCAAGATCACAGATTTTGGG CTGGCCAAA CTGCTGGGTGCGGAAGAGAAAG AATACCATGCAGAAGG AGGCAAAGTAAGGAG GTGGCTTTAGGTCAGCCAGCATTT TCCTGA	73
6	Bio-Rad	dHsa CP20 00039	EGFR	7p12	7:55242438-55242560	c.2235_2249del15	E746-A750del	GAAAGTTAAAATTCCCGTCGCTATCAAGGAAT TAAGAGAAG CAACATCTCCGAAAGCCAACAA GGAAATCCTCGATGTGAG TTTCTGCTTTGCTG TGTGGGGGTCCATGGCTCTGAACCT CAG	78
7	Bio-Rad	dHsa CP20 00019	EGFR	7p12	7:55249042-55249164	c.2369C>T	T790M	CTGCCTCACCTCCACCGTGCAGCTCATCACG CAGCTCAT GCCCTTCGGCTGCCTCCTGGACT ATGTCCGGGAACACAA AGACAATATTGGCTCC CAGTACCTGCTCAACTGGTGTGT GCAGAT	80

SUPPLEMENTARY TABLE S2.1 COTNINUED.

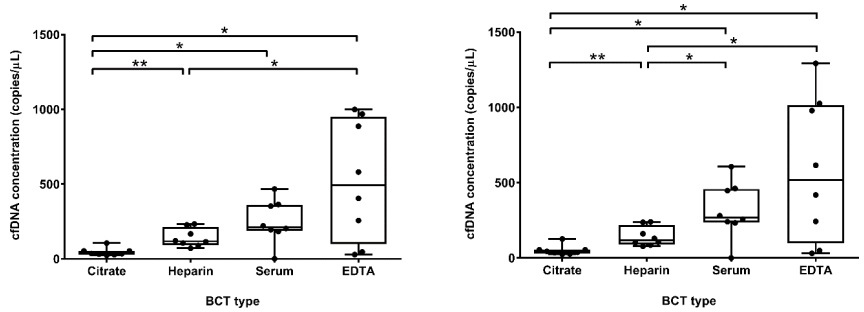
Assay	Supplier	ID	Gene	Chromosome mapping	Chromosome location	variant	Protein change	Amplicon Context [FAM/HEX]	Amplicon length
8	Bio-Rad	dHsaI S2504192	EGFR	7p12	7:55241679-55241801	c.2155G>A	G719S	AACTGAATTCAAAAAGATCAAAGTGCTGGGCT CCGGTGC GTTCGGCACGGTGTATAAGGTAAG GTCCCTGGCACAGG CCTCTGGGCTGGGCCG CAGGGCCTCTCATGGTCTGGT GGGGAGCCC	65
9	Bio-Rad	dHsa CP2000043	EGFR	7p12	7:55259493-55259615	c.2582T>A	L861Q	GTCAAGATCACAGATTTTGGGCTGGCCAAACT GCTGGGT GCGGAAGAGAAAGAATACCATGCA GAAGGAGGCAAAGTA AGGAGGTGGCTTTAGG TCAGCCAGCATTTTCCTGACAC CAGGGAC	64
10	Bio-Rad	dHsa MDV2510584	KRAS	12p12.1	12:25398224-25398346	c.34G>T	G12C	ATTATTTTTATTATAAGGCCTGCTGAAAATGAC TGAATATA AACTTGTGGTAGTTGGAGCT[G/T]G TGGCGTAGGCAAGA GTGCCTTGACGATACAG CTAATTCAGAATCATTTTGTG GACGAATA	57

SUPPLEMENTARY TABLE S2.2. KRAS G12/G13 Screening Multiplex Assay

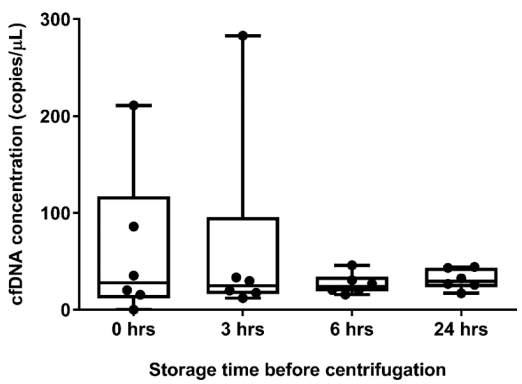
Assay	Supplier	Gene	Chromosome mapping	ID	Chromosome location	variant	Protein change	Amplicon Context [FAM/HEX]	Amplicon length
11	Bio-Rad	KRA S	12p12.1	dHsaMDV2510586	12:25398223-25398345	c.35G>C	G12A	TTATTTTTATTATAAGGCCTGCTGAAAA TGACTGAATATAAAC TTGTGGTAGTTG GAGCTG[G/C]TGGCGTAGGCAAGAGTG CCTTGACGATACAGCTAATTCAGAATC ATTTTGTGGACGA ATAT	57
				dHsaMDV2510584	12:25398224-25398346	c.34G>T	G12C	ATTATTTTTATTATAAGGCCTGCTGAAA ATGACTGAATATAA ACTTGTGGTAGTT GGAGCT[G/T]GTGGCGTAGGCAAGAGT GCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACG AATA	57

SUPPLEMENTARY TABLE S2.2 COTNINUED.

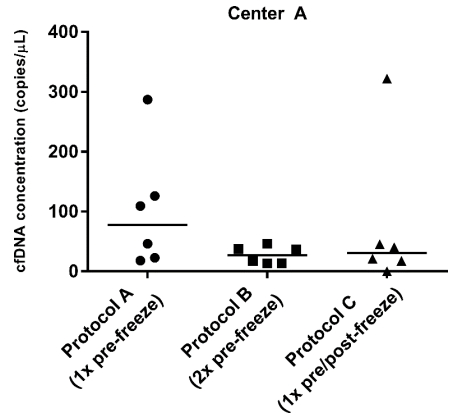
Assay	Supplier	Gene	Chromosome mapping	ID	Chromosome location	variant	Protein change	Amplicon Context [FAM/HEX]	Amplicon length
				dHsaMDV2510596	12:25398223-25398345	c.35G>A	G12D	TTATTTTTATTATAAGGCCTGCTGAAAA TGACTGAATATAAAC TTGTGGTAGTTG GAGCTG[G/A]TGGCGTAGGCAAGAGTG CCTTGACGATACAGCTAATTCAGAATC ATTTTGTGGACGA ATAT	57
				dHsaMDV2510590	12:25398224-25398346	c.34G>C	G12R	ATTATTTTTATTATAAGGCCTGCTGAAA ATGACTGAATATAAA CTTGTGGTAGTT GGAGCT[G/C]GTGGCGTAGGCAAGAGT GCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACG AATA	57
				dHsaMDV2510588	12:25398224-25398346	c.34G>A	G12S	ATTATTTTTATTATAAGGCCTGCTGAAA ATGACTGAATATAAA CTTGTGGTAGTT GGAGCT[G/A]GTGGCGTAGGCAAGAGT GCCTTGACGATACAGCTAATTCAGAAT CATTTTGTGGAC GAATA	57
				dHsaMDV2510592	12:25398223-25398345	c.35G>T	G12V	TTATTTTTATTATAAGGCCTGCTGAAAA TGACTGAATATAA ACTTGTGGTAGTTG GAGCTG[G/T]TGGCGTAGGCAAGA GTG CCTTGACGATACAGCTAATTCAGAATCATTTTGT GGACGAATAT	57
				dHsaMDV2510598	12:25398220-25398342	c.38G>A	G13D	TTTTTATTATAAGGCCTGCTGAAAATGA CTGAATATAAACTT GTGGTAGTTGGAG CTGGTG[G/A]CGTAGGCAAGAGTGCCT TGACGATACAGCTAATTCAGAATCATTTTGTGGACGAAT ATGAT	57



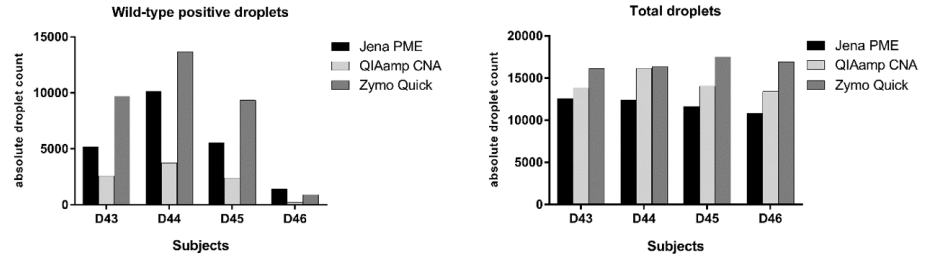
SUPPLEMENTARY FIGURE S1. The boxplots indicate cfDNA concentrations as shown on the y-axis, while comparing citrate, heparin, serum, and EDTA BCTs from 8 healthy controls as shown on the x-axis. The crossing lines indicate medians, the upper and lower limits of the boxes indicate interquartile ranges (25^{th}/75^{th} percentiles), and whiskers represent minima and maxima. Using assay 2 (A), significantly higher cfDNA concentrations were found in EDTA samples compared to paired heparin samples and citrate samples (493.0 [98.7 – 948.5] vs. 117.5 [93.3 – 212.0] and 35.1 [28.5 – 53.3], both $p < 0.05$). Compared to citrate samples, median cfDNA concentrations in both paired serum and heparin samples were significantly higher (212.0 [186.0 – 361.3] and 117.5 [93.3 – 212.0] vs. 35.1 [28.5 – 53.3], $p < 0.01$ and $p < 0.05$). Using assay 3 (B), significantly higher cfDNA concentrations were found in EDTA samples compared to paired heparin and citrate samples (516.5 [97.7 – 1014.0] vs. 117.5 [89.0 – 218.0] and 40.6 [29.4 – 54.0], both $p < 0.05$). Furthermore, serum samples contained significantly higher cfDNA concentrations compared to paired heparin and citrate samples (269.0 [235.0 – 457.5] vs. 117.5 [89.0 – 218.0] and 40.6 [29.4 – 54.0], both $p < 0.05$), as well as heparin samples contained significantly higher cfDNA concentrations compared to citrate samples (117.5 [89.0 – 218.0] and 40.6 [29.4 – 54.0], $p < 0.01$). *P < 0.05, **P < 0.01, ***P < 0.001).



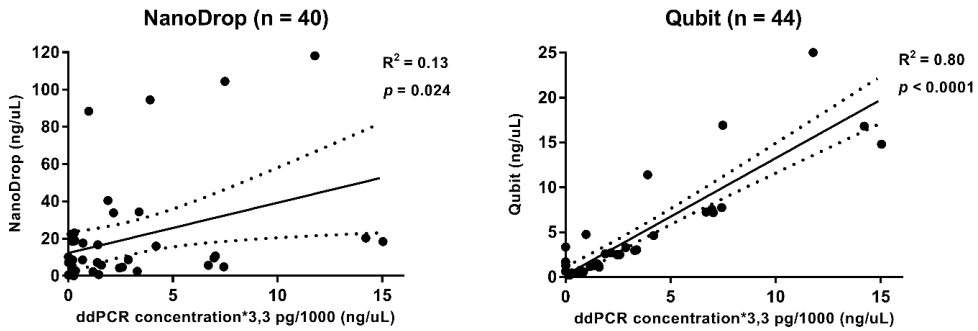
SUPPLEMENTARY FIGURE S2. Influence of storage time until centrifugation on cfDNA concentrations in paired EDTA samples from 6 healthy individuals after PCR using assay 3. Time points T1-T4 are shown on the x-axes and median cfDNA concentrations on the y-axes. No significant differences were found between median DNA concentrations at consecutive time points T1-T4.



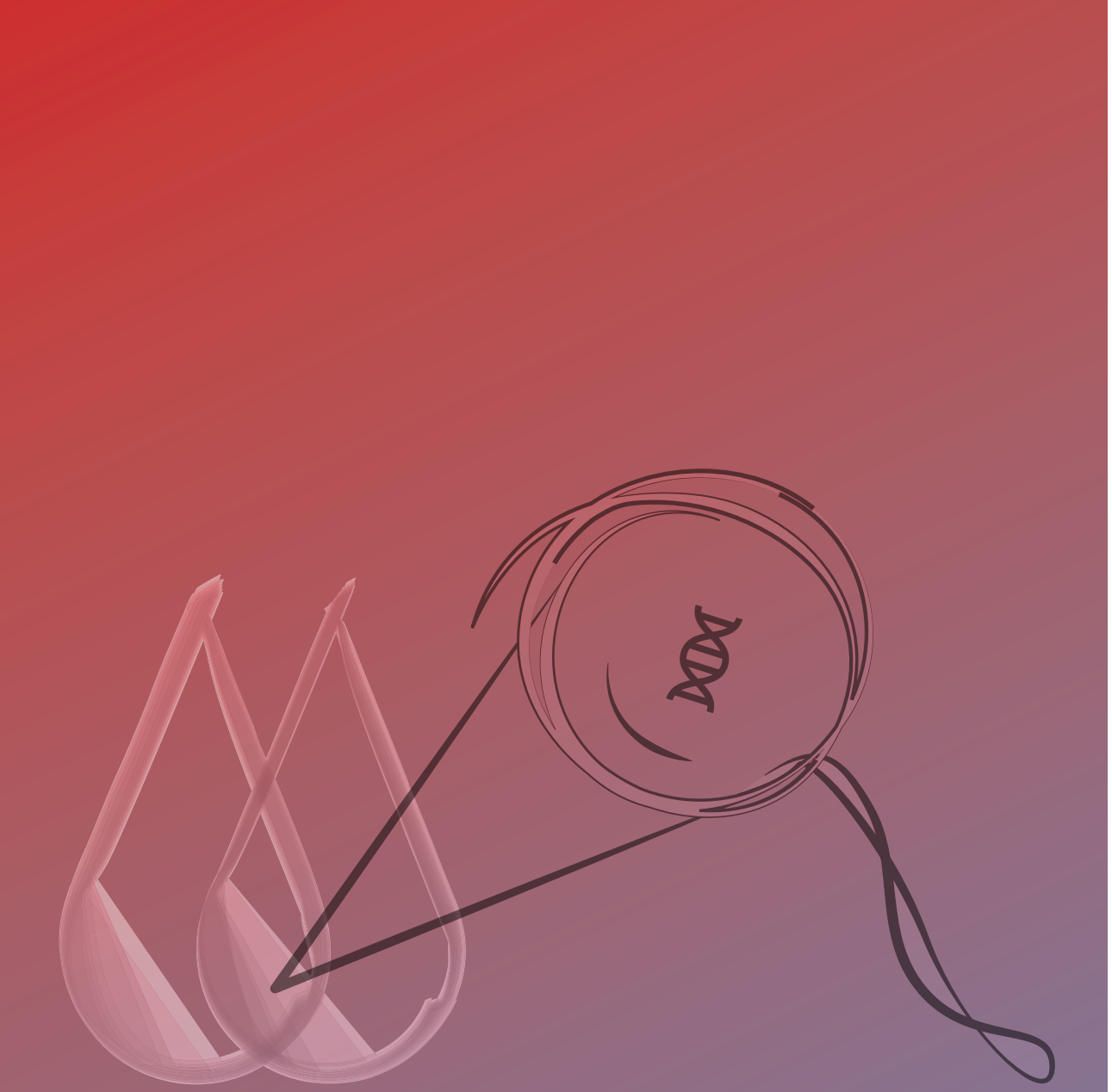
SUPPLEMENTARY FIGURE S3. Additional comparison of centrifugation protocols A-C in EDTA samples from D12-D17 show similar results using assay 3, validating the results of this experiment using assay 2: median cfDNA concentrations detected after centrifugation using protocol A were 77.5 (21.6 – 166.3) copies/µl, compared to 27.1 (13.6 – 39.6) copies/µl using protocol B and 30.8 (13.3 – 114.5) copies/µl using protocol C.



SUPPLEMENTARY FIGURE S4. Isolation methods in healthy individuals using assay 4. Healthy individuals (D43-D46) are depicted on the x-axes. Absolute droplet counts are shown on the y-axes for both wild-type (A) and total positive droplet (B). The gray scaled bars represent 3 different commercially available isolation kits.



SUPPLEMENTARY FIGURE S5. DNA quantification of EDTA samples prior to ddPCR using assay 1. Forty-four samples were quantified using both methods. After excluding 4 quantification results from analysis due to negative values, NanoDrop resulted in R^2 of 0.13 (β= 2.87[CI 0.65 - 5.10], $p < 0.05$). For Qubit, R^2 was 0.80 (β = 1.30[CI 1.10 - 1.50]), $p < 0.0001$) for ddPCR.



6

Conceptual basis and clinical rationale for tumor DNA detection in plasma and saliva from head and neck cancer patients undergoing (chemo)radiation

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Work in progress

ABSTRACT

Background

Head and neck squamous cell carcinoma (HNSCC) recurrence rates are high after chemo(radiation), while salvage surgery is not always possible and associated with increased complication rates. Cell-free tumor DNA in plasma and saliva from HNSCC patients may serve as a diagnostic biomarker for early identification of non-responders to primary (chemo) radiation in order to timely adjust treatment.

Methods

Using droplet digital PCR (ddPCR), tumor DNA levels were measured in plasma and saliva from 5 HNSCC patients before and during (chemo)radiation at 5 time points. Magnetic resonance imaging (MRI) was performed additionally in 2 patients to estimate actual tumor burden, described as gross tumor volume (GTV).

Results

Both patients who developed recurrent disease at later stage, showed a decline in plasma tumor DNA concentrations and GTV over the course of treatment, while salivary tumor DNA either increased or remained undetected. The other 3 patients who remained free of clinical recurrence, had various plasma and salivary tumor DNA concentrations.

Conclusion

We illustrate the practical feasibility and importance of a streamlined clinical workflow as a model for serial liquid biopsies and MRI during (chemo)irradiation in HNSCC patients. The interpretation of tumor DNA levels in plasma and saliva will be investigated in the near future.

INTRODUCTION

The overall 5-year survival rate of head and neck squamous cell carcinoma (HNSCC) patients in The Netherlands ranges from only 39% to 66% depending on primary tumor site and stage. The 5-year pathology proven loco-regional recurrence rate is only 20-29% [1]. Salvage surgery is generally considered as the only treatment option with curative intent in case of (loco)regional recurrence after (chemo)radiation. However, not all (chemo)irradiated patients may benefit from salvage surgery as its success strongly depends on factors such as site, extent of relapse and patient performance status, while complication rates are high [2, 3]. Therefore, a more personalized disease management is desired by early identification of non-responders to (chemo)radiation in order to cease ineffective treatment and timely adjust treatment regimen.

More accurate and tumor-specific methods for the early detection of minimal residual or recurrent disease are under investigation. In particular the detection of cell-free circulating tumor DNA (ctDNA) in blood from cancer patients (liquid biopsy) is of great interest, as it possibly better reflects cancer biology rather than morphological changes as observed by routine diagnostics. To date, research results on highly sensitive quantification of ctDNA in plasma from HNSCC patients are promising [4]. Principles and clinical applications of ctDNA detection are based on its potential diagnostic, predictive, and prognostic value. The strategy to identify biomarkers in saliva for the early detection and prognosis of HNSCC has not yet proven to be consistently effective [5]. In this study, we focus on the quantification of *TP53* mutant cell-free DNA in blood and saliva samples from (chemo)irradiated HPV-negative HNSCC patients using ultrasensitive droplet digital PCR (ddPCR) for future diagnostic purpose. We show preliminary results that are part of an ongoing prospective observational pilot study, in order to illustrate the conceptual basis and rationale of a streamlined clinical workflow for the detection and quantification of tumor DNA in plasma and saliva of HNSCC patients undergoing (chemo)radiation. Therefore, we collected samples on 5 predetermined time points from 5 HNSCC patients before, during and after primary (chemo)radiation, and performed repeated MRI scans in 2 patients.

MATERIALS AND METHODS

Study participants

Primary HNSCC patients were recruited from July 2017 until November 2018. All patients consented to an institutional review board-approved protocol (NL57164.041.16) permitting the collection and analysis of plasma and saliva samples pretreatment and during treatment

with or without additional MRI imaging of the primary tumor, as well as the collection of clinicopathological and radiological data from hospital charts. The latter included a diagnostic MRI. Next-generation sequencing (NGS) analysis of primary tumor samples was required in order to retrieve its mutational status before definitive enrollment. Patients were eligible for study participation in case of histologically confirmed HNSCC, primary tumor stage T2-T4, *TP53* mutant-positive primary tumor sample, and when scheduled for primary (chemo)radiation with curative intent.

Treatment monitoring protocol

Patients were enrolled for serial collection of plasma and saliva before and during (chemo) radiation. Baseline sampling took place 7–10 days pretreatment (T0). Subsequent monitoring sampling took place weekly during the 2nd–5th week of radiotherapy, usually within 10–20 minutes around fractions at the radiotherapy department of our institution (T1–T4, corresponding with treatment week 2–5 respectively). Patients who consented for additional magnetic resonance imaging (MRI) assessment during treatment, also underwent MRI at T1–T4, in order to simultaneously monitor tumor burden, described as gross tumor volume (GTV).

Sample workup

All primary tumor samples were formalin fixed paraffin embedded (FFPE) incisional or excisional biopsy specimen, microscopically containing >30% tumor cells. In one case, only FFPE of a lymph nodal fine-needle aspiration biopsy specimen was available for mutational analysis. In order to confirm *TP53* mutational status of primary tumor samples, targeted NGS analysis was performed on DNA samples that were created by DNA isolation from the corresponding diagnostic FFPE samples using COBAS DNA Sample Preparation Kit (Roche, Basel, Switzerland). NGS was performed using the Ion Torrent™ PGM platform and the Cancer Hotspot Panel v2+ (Thermo Fisher Scientific, Waltham, MA, USA), as described previously [8].

Blood samples were collected in 10 ml K2EDTA blood collection tubes (BD Vacutainer, Franklin Lakes, NJ, USA). Centrifugation took place for 10 min at 800 g (Rotina 380, Hettich, Germany), followed by microcentrifugation of 1 minute at 11,000g (5424 Microcentrifuge, Eppendorf, Germany) in order to spin off cellular debris. Subsequently, supernatant plasma was aliquoted in 1 mL portions and stored at -80°C until DNA isolation. Whole saliva samples were collected in 0.8-1.4 mL Salivette Cortisol tubes (Sarstedt, Nümbrecht, Germany). Centrifugation took place for 5 minutes at 5,000 g, after which the samples were stored at 4°C. Cell-free DNA (cfDNA) was isolated from 1.0 – 2.0 mL of plasma and 0.35 – 1.70 mL of saliva using Zymo Quick cfDNA serum & plasma kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. All isolated DNA samples were eluted in 35 µl elution buffer as provided with the kit and stored at 4°C until ddPCR analysis.

DNA quantity measurement of isolated DNA samples took place using a Qubit fluorometer with double stranded DNA (dsDNA) High Sensitivity Assay Kit (Thermo Fisher Scientific). The COBAS isolated DNA samples functioned as positive control samples for each assay. No Template Controls (NTC) were used to control for environmental contamination. Wild-type-only (WT-only) samples were used to estimate false-positive rates, and were created by isolating cfDNA from plasma (6 mL) and saliva (2.5 mL) from anonymous healthy volunteers using the Zymo Quick cfDNA serum & plasma kit according to manufacturer instructions.

Assay validation and experimental setup

Plasma samples from all patients were analyzed for *TP53* mutations, identified in the primary tumor tissue by NGS. Mutant and Wild type *TP53* sequences were used as DNA template for designing ddPCR (Bio-Rad Laboratories, Hercules, CA, USA) assays according to Minimum Information for Publication of Quantitative Digital PCR Experiments (MIQE) guidelines (supplementary Table S1) [6]. Assay performance was verified by comparing "Variant Allele Frequency" (VAF) to "Fractional Abundance" (FA) as measured in positive control samples by NGS and ddPCR, respectively. Optimal annealing temperature was determined by running a temperature gradient according to manufacturer instructions. Quality assurance and false-positive rate estimation was carried out as described previously [6]. DdPCR reaction volumes of 22 µl were prepared, consisting of 16 µl mastermix (11 µl Supermix for Probes [no deoxyuridine triphosphate], 1 µl of primer/probe mix for both MT and WT *TP53*, and additional 4 µl of purified H2O), and 6 µl of patient cfDNA sample. One µl of positive control samples was added to reaction mixes. The NTCs contained 10 µl of purified Milli-Q water (MilliporeSigma, Burlington, Massachusetts, USA) instead of DNA sample. WT-only samples contained 6 µl of cfDNA. From the PCR reaction mixture, 20 µl was used for emulsification using the Automated Droplet Generator (Bio-Rad Laboratories). Thermal cycling conditions were set at 95°C for 10 min (1 cycle), 95 °C for 30 s and 55°C for 60 s (40 cycles), 10 minutes 98°C (1 cycle), and infinite hold at 12°C. DdPCR analysis was performed using the QX200 ddPCR system and QuantaSoft v1.7.4.01917 software (Bio-Rad Laboratories). To ensure experiment quality, wells containing a total droplet count of less than 10,000 would be considered invalid and excluded from analysis.

RESULTS

Clinicopathological characteristics

Five patients (median age 59 [56 – 82] years) with advanced stage IV HPV-negative HNSCC (ranging T2N2bM0–T4aN3bM0) were enrolled (A-E). Three patients were male. All patients were current smokers (32–100 pack years), and 4 patients had a history of alcohol consumption (7–70 units/week). Four patients had an oropharyngeal carcinoma and 1 patient had a hypopharyngeal carcinoma, with GTV ranging from 9.7–15.5 cm^3 on pretreatment diagnostic MRI (Figure 1 and Table 1). No patients were diagnosed with HNSCC previously.

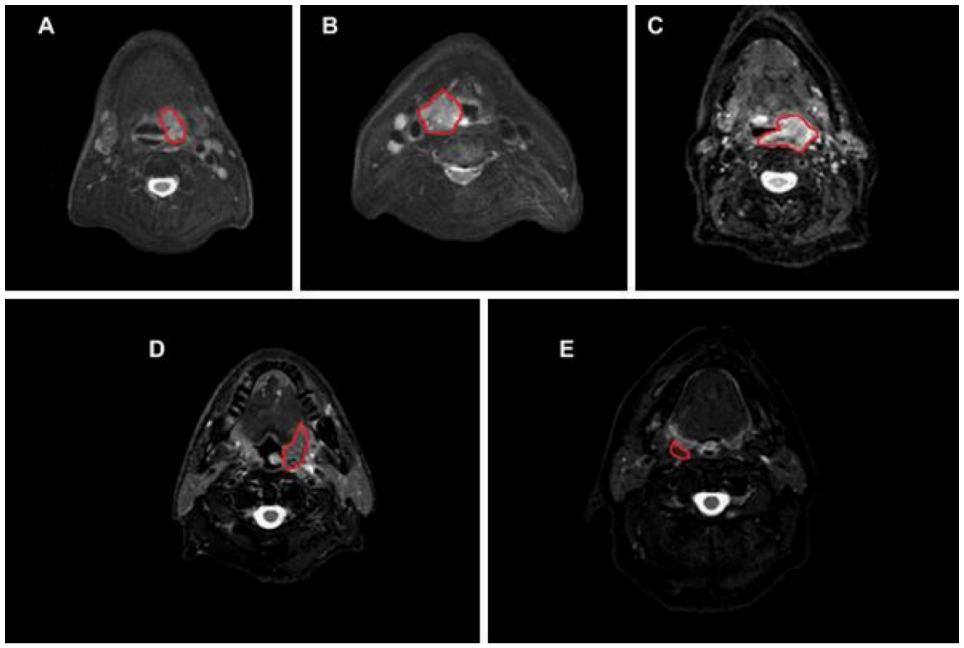


FIGURE 1. Diagnostic T2-weighted MRI of the primary tumor (demarcated by red lines) of all five patients acquired before start of treatment.

Histopathologically, there was no evidence of vascular invasion in any patient. In patient B, mutations were identified in 3 different *TP53* regions in the primary tumor biopsy specimen. Of these mutations, the one with the highest VAF was selected as mutant target for ctDNA quantification (Supplementary Table S2).

TABLE 1. Patient characteristics

Subject ID	Age	Sex	Smoking (pack years)	Alcohol (units/week)	Biopsy type	Tumor site	TNM-stage	Treatment received
A	57	M	100	70	Incisional	OPSCC	T2N3bM0	RTx + CTx
B	56	M	37	3	Incisional	HSCC	T4aN3bM0	RTx + CTx
C	82	F	34	7	Cytology	OPSCC	T2N2cM0	RTx
D	59	M	55	0	Incisional	OPSCC	T3N3bM0	RTx + CTx
E	60	F	32	7	Excisional	OPSCC	T2N2bM0	RTx

OPSCC, Oropharynx squamous cell carcinoma; HSCC, Hypopharyngeal squamous cell carcinoma; RTx, radiotherapy; CTx, chemotherapy

Treatment

All patients received radiotherapy (70 Gy in 35 fractions). Two patients (A and D) received concomitant cisplatin (three courses of three weekly 100 mg/m^2), and 1 patient (B) received only one dose of cisplatin (100 mg/m^2) and carboplatin (250 mg/m^2) each due to impaired renal function and thrombocytopenia, respectively (Supplementary Table S2.1).

Follow-up

Patient A developed locoregional recurrent disease 10 months after treatment, and patient B died due to recurrent tumor-associated complications 4 months after treatment. These two patients underwent additional weekly MRI assessment during radiotherapy. Patients C,D and E remained free of locoregional or distant disease at least the first year of follow-up.

Assay validation

TP53 mutations were all detected optimally by ddPCR at 55°C in FFPE from all patients as shown by the temperature gradient we performed for each mutant assay (Supplementary Figure S1). FA of MT copies ranged from 17–71% in positive control samples as measured by ddPCR, compared to VAF MT template percentages of 17– 60% as measured by NGS. False-positive rate estimation was necessary to determine aspecific MT signal. MT-false-positive droplets were detected in WT-only plasma and/or saliva samples (0–102.7 copies/mL) with all assays, except for assay B (Supplementary Figure S2). The only 2 WT-false-positive droplets were detected in NTCs with assay C, while no MT-positive droplets were detected at all (Supplementary Figure S3). All validation results are summarized in Supplementary Table S3.

Serial analysis of patient samples

Blood and saliva samples were collected from all patients on all time points. CfDNA was quantified in collected samples from all 5 patients (Figure 2). One blood sample from patient

A collected on T3 and was missing due to loss in transit. MT copies of *TP53* were detected in plasma from all patients at least once during serial analysis, ranging from 0–603 copies/ml plasma (Supplementary Figures S4). MT copies of *TP53* were detected in saliva from 3 patients (A, D, and E) at least once, ranging from 0–957 copies/ml saliva (Supplementary Figures S4). Pretreatment plasma from 3 patients (A, B, and E) was MT-positive, while only pretreatment saliva from D was MT-positive.

Serial imaging of primary tumor

Serial imaging was performed on all time points for patients A and B. For patient A, GTV declined from 15.5 cm^3 at baseline to 1.8 cm^3 at week 5 of treatment (T4). The tumor of patient B initially increased from a baseline GTV of 12.8 cm^3 to 17.6 cm^3 at the second week of treatment (T1), after which it declined to 1.7 cm^3 at week 5 of treatment (Figure 2).

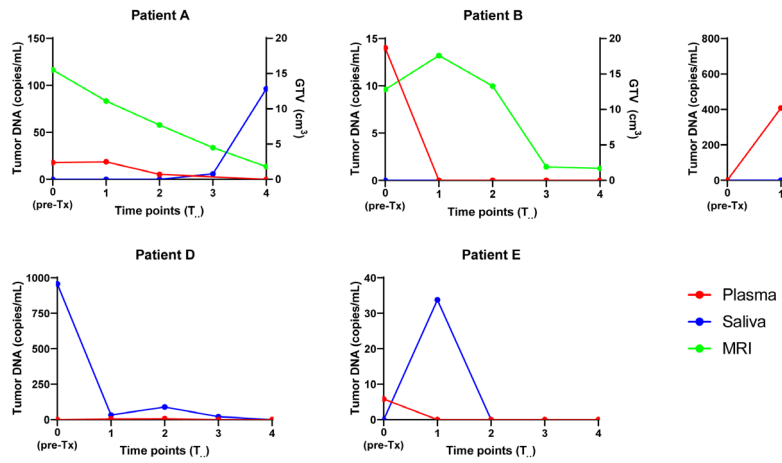


FIGURE 2. Plasma and saliva tumor DNA levels (copies/ml) measured in HNSCC patients before (Pre-Tx) and during (chemo)radiation at corresponding time points, as shown on x-axes. Red lines represent plasma concentrations and blue lines represent saliva concentrations. Note that y-axes are fitted to tumor DNA concentrations, which varied considerably between patients. Additional serial GTV measurements using MRI during treatment of patient A and B are shown in green, and are associated with right y-axes.

DISCUSSION

Monitoring treatment response using ctDNA analysis in head and neck cancer patients during and after treatment is not as vastly investigated as other types of cancer such as breast, colorectal, and lung cancer [7]. Its potential to improve survival of HNSCC patients in the future should encourage to be investigated more intensively [8]. Our preliminary results show that serial assessment of tumor DNA in plasma and saliva from HNSCC patients is feasible. However, its relevance in predicting treatment response during definitive (chemo) radiation has not been clarified yet. We observed different patterns in fluctuation of cell-free tumor DNA concentrations in both simultaneously collected plasma and saliva samples. On the one hand, an initial rise occurred in ctDNA concentration in plasma from patient C in week 2 of treatment, while a similar rise was seen in saliva from patient E. Variable release of tumor DNA due to radiation-induced cell death might have caused different fluctuation patterns in tumor DNA concentration among patients, as the release of DNA fragments in blood depends on both rate and extent of necrosis and apoptosis [13]. Lo et al. demonstrated that in nasopharyngeal carcinoma patients treated with definitive radiotherapy, plasma EBV-DNA concentrations increased during the first week of treatment and subsequently declined. They speculated this to happen due to a combination of an increase in cell death and decreasing tumor burden, respectively [9]. On the other hand, no initial peak was observed at all for patient A, B, and D, which could be explained by differences in net result of gradual decrease in tumor burden as shown by a steady decline in GTV in patient A. Similar results are reported for systemically treated HPV-positive HNSCC patients [10-12]. Strikingly, the most advanced stage HNSCC patient did not had any detectable tumor DNA along the course of treatment, while GTV initially increased. Whether this is the result of therapy-resistant tumor cells (e.g. lack of radiosensitivity) or shortcomings in ctDNA analysis remains unclear. Technically, our validation showed that some of the used assays were prone to false-positives. However, this might have caused only subtle differences in absolute tumor DNA concentrations, but no changes in fluctuation patterns on the whole.

Another relevant factor is the estimated half-life of ctDNA in blood circulation of approximately 2 hours [14], implying that mainly tumor cells that died less than 2 hours prior to sample collection contribute to ctDNA levels. Although less is known about the half-life of cell-free DNA in saliva, recent in vitro experiments showed a 50% reduction of salivary DNA fragments in about 3 hours at 37°C [15]. In our study, post-fraction sampling of both plasma and saliva took place within 20 minutes after radiation fractions. However, instant rise of tumor DNA levels following radiation fractions seems unlikely, as in most solid tumors radiation-induced cell death starts only days after a fraction [16]. Changes in tumor DNA concentrations further appeared to vary independently between matched plasma and saliva. Most interestingly, tumor DNA concentration in saliva from the OPSCC

patient that eventually developed recurrence rose towards the end of treatment, while ctDNA concentration in matched plasma samples dropped after start of treatment. The exact mechanism of tumor DNA release into saliva has not been fully understood yet, but it is likely that necrotic HNSCC cells shed DNA directly into saliva. However, patient E still had tumor DNA positive saliva after excisional biopsy with clear margins, which can only be explained by shedding of plasma ctDNA in saliva through the salivary glands. Wang et. al. reported a higher sensitivity of tumor DNA detection in saliva from OSCC patients than from patients with HNSCC in other sites, indicating the shedding of tumor DNA directly into saliva. They also found that combined tumor DNA analysis of saliva and matched plasma samples yielded higher overall detection rates for all HNSCC patients compared to analysis of solely plasma [17].

Besides treatment response prediction, tumor-specific posttreatment disease monitoring is desired as well, since the first 2 years of follow-up are critical due to high risk of recurrence for which active surveillance of HNSCC patients is strongly recommended [18]. Recent reports on posttreatment disease monitoring using PCR-based NGS analysis showed that plasma ctDNA is detectable prior to treatment in patients with stage IV disease and that detectable ctDNA in plasma from curatively treated patients may serve as a biomarker for local recurrence prior to clinical manifestation [19]. Additionally, results from an observational study on disease stratification and prognosis demonstrated plasma ctDNA copy number instability to be a superior predictor for overall survival compared to lymph node involvement [20]. Apart from monitoring mutant *TP53*, approximately 20% of OPSCC cases are being caused by human papilloma virus (HPV) infection, which is considered to be the etiological counterpart of smoking and alcohol consumption [21]. Circulating tumor DNA shed from HPV-positive tumors is similarly quantifiable in blood and saliva by targeting HPV E6/E7 gene regions[22]. Although no HPV-positive HNSCC patients were included in our cohort yet, inclusion is planned for further analysis in the future.

In conclusion, we illustrate the practical feasibility and importance of a streamlined clinical workflow as a model for the detection of mutant *TP53* tumor DNA in simultaneously collected plasma and saliva samples, and serial MRI during (chemo)irradiation in HNSCC patients. The interpretation of tumor DNA levels in plasma and saliva for clinical purpose requires further exploration and will be investigated in the near future.

REFERENCES

1. de Ridder M, Balm AJM, Baatenburg de Jong RJ, Terhaard CHJ, Takes RP, Slingerland M, et al. Variation in head and neck cancer care in the Netherlands: A retrospective cohort evaluation of incidence, treatment and outcome. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2017;43:1494-502.
2. Putten L, Bree R, Doornaert PA, Buter J, Eerenstein SE, Rietveld DH, et al. Salvage surgery in post-chemoradiation laryngeal and hypopharyngeal carcinoma: outcome and review. Acta Otorhinolaryngol Ital. 2015;35:162-72.
3. Elbers JBW, Al-Mamgani A, van den Brekel MWM, Jozwiak K, de Boer JP, Lohuis P, et al. Salvage Surgery for Recurrence after Radiotherapy for Squamous Cell Carcinoma of the Head and Neck. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 2018:194599818818443.
4. van Ginkel JH, Slieker FJB, de Bree R, van Es RJJ, Van Cann EM, Willems SM. 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 oncology. 2017;75:8-15.
5. Arantes L, De Carvalho AC, Melendez ME, Lopes Carvalho A. Serum, plasma and saliva biomarkers for head and neck cancer. Expert review of molecular diagnostics. 2018;18:85-112.
6. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, et al. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin Chem. 2013;59:892-902.
7. Pantel K, Alix-Panabieres C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. Nature reviews Clinical oncology. 2019.
8. Nonaka T, Wong DTW. Liquid Biopsy in Head and Neck Cancer: Promises and Challenges. Journal of dental research. 2018;97:701-8.
9. Lo YM, Leung SF, Chan LY, Chan AT, Lo KW, Johnson PJ, et al. Kinetics of plasma Epstein-Barr virus DNA during radiation therapy for nasopharyngeal carcinoma. Cancer research. 2000;60:2351-5.
10. 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. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2018.
11. Hanna GJ, Lau CJ, Mahmood U, Supplee JG, Mogili AR, Haddad RI, et al. Salivary HPV DNA informs locoregional disease status in advanced HPV-associated oropharyngeal cancer. Oral oncology. 2019;95:120-6.
12. Chera BS, Kumar S, Beaty BT, Marron D, Jefferys SR, Green RL, et al. Rapid Clearance Profile of Plasma Circulating Tumor HPV Type 16 DNA during Chemoradiotherapy Correlates with Disease Control in HPV-Associated Oropharyngeal Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2019.
13. 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 research. 2001;61:1659-65.
14. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor

dynamics. Nature Medicine. 2008;14:985-90.

15. Yao W, Mei C, Nan X, Hui L. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. Gene. 2016;590:142-8.
16. Eriksson D, Stigbrand T. Radiation-induced cell death mechanisms. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2010;31:363-72.
17. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Science translational medicine. 2015;7:293ra104.
18. Mehanna H, Wong WL, McConkey CC, Rahman JK, Robinson M, Hartley AG, et al. PET-CT Surveillance versus Neck Dissection in Advanced Head and Neck Cancer. The New England journal of medicine. 2016;374:1444-54.
19. Egyud M, Sridhar P, Devaiah A, Yamada E, Saunders S, Stahlberg A, et al. Plasma circulating tumor DNA as a potential tool for disease monitoring in head and neck cancer. Head & neck. 2019;41:1351-8.
20. 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;64:959-70.
21. Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nature reviews Cancer. 2018;18:269-82.
22. Ahn SM, Chan JY, Zhang Z, Wang H, Khan Z, Bishop JA, 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;140:846-54.

SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE S1. Mutations and assays

Subject ID	Gene	Mutation	Mutation effect	Protein change	Assay ID	MIQE Context [wt/mut]
A	*TP53*	c.817C>T	missense	Arg273Cys	A	TTGCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTA CTGGGACGGAACAGCTTTGAGGTG[C/T]GTGTTTGT GCCTGTCCTGGGAGAGACCGGCGCACAGAGGAAG AGAATCTCCGCAAGAAAGG
B	*TP53*	c.976G>T	nonsense	Glu326*	B	CCTAGCACTGCCCAACAACACCAGCTCCTCTCCCC AGCCAAAGAAGAAACCACTGGATGGA[G/T]AATATTT CACCCTTCAGGTACTAAGTCTTGGGACCTCTTATCA AGTGGAAAGTTTCCAGTC
C	*TP53*	c.527G>A	missense	Cys176Tyr	C	CGCGTCCGCGCCATGGCCATCTACAAGCAGTCACA GCACATGACGGAGGTTGTGAGGCGCT[G/T]CCCCC ACCATGAGCGCTGCTCAGATAGCGATGGTGAGCAG CTGGGGCTGGAGAGACGACAG
D	*TP53*	c.421_430del	frameshift	Cys141Serfs*26	D	CTTCCTCTTCCTACAGTACTCCCCTGCCCTCAACAA GATGTTTTGCCAACTGGCCAAGACC[TGCCCTGTGC/ -]AGCTGTGGGTTGATTCCACACCCCCGCCCGGCAC CCGCGTCCGCGCCATGGCCATCTACAA
E	*TP53*	c.455insC	frameshift	Pro153Alafs*28	E	AGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTG CAGCTGTGGGTTGATTCCACACCCCC[/C]GCCCGGC ACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTC ACAGCACATGACGGAGGTT

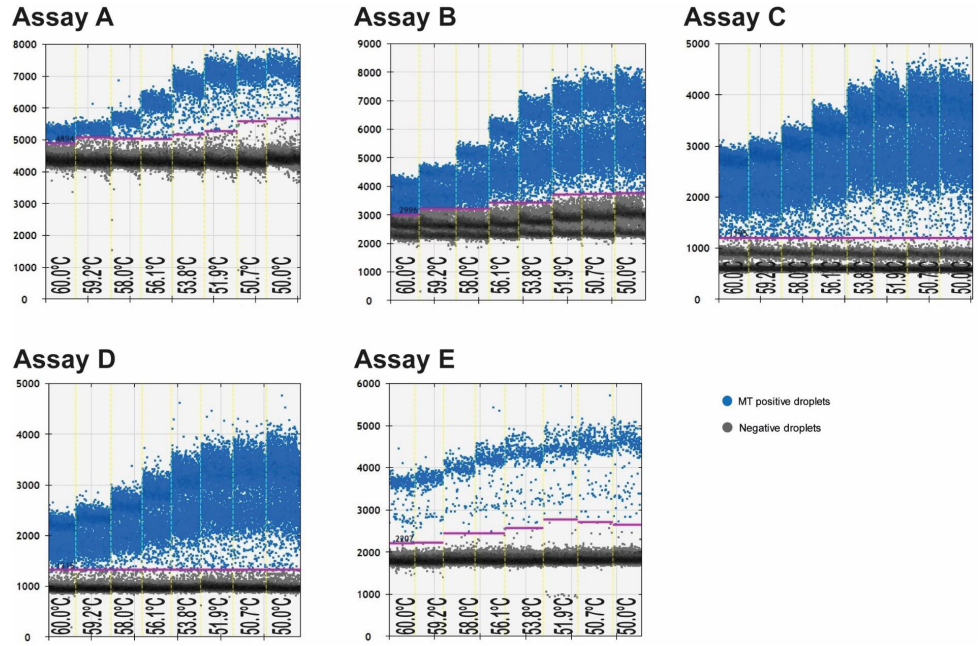
SUPPLEMENTARY TABLE S2 Tumor characteristics

Subject ID	Max primary tumor diameter (mm)	Differentiation grade	Growth type	Vascular invasion	Mutation	Genomic region (exon)
A	37	Moderate	Endophytic	No	c.817C>T	8
B	25	Moderate	Spiculated	No	c.976G>T c.1037A>C c.815del29	9 10 8
C	29	Moderate	N/A	N/A	c.527G>A	5.2
D	46	Moderate	Endophytic	Unclear	c.421_430del	5.1
E	20	Poor	Spiculated	No	c.455insC	5.1

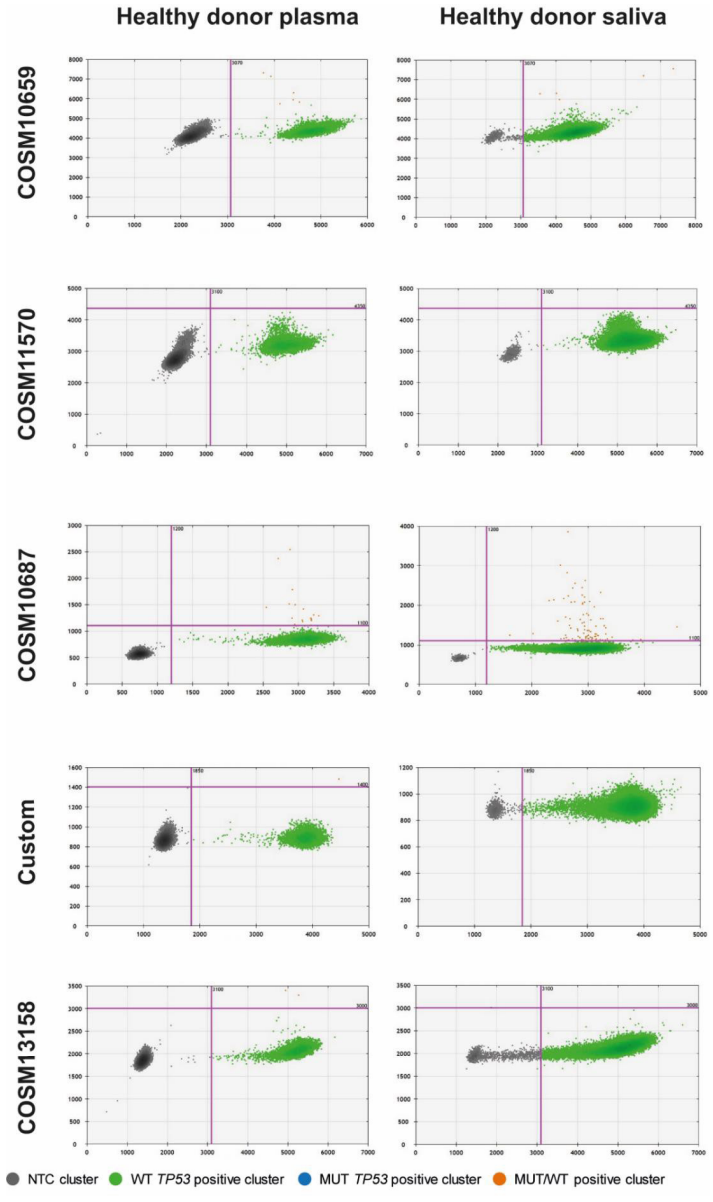
SUPPLEMENTARY TABLE S3. Assay validation

Sample type	Sample matrix	Value	Assays				
			A	B	C	D	E
MT-positive controls	Tissue	VAF (%)	60	22	55	52	17
	Tissue	FA (%)	71	21	58	49	17
	Tissue	Fluorescence threshold (MUT/WT)	5360/3070	4350/3100	1300/1200	1400/1850	3000/3100
WT-only-controls	Plasma	Mean false-positive concentration (copies/mL)	2.5	0	7.0	0.4	0.7
	Saliva	Mean false-positive concentration (copies/mL)	6.5	0	102.7	0	0
NTC	H_2O	Mean # positive droplets (MUT/WT)	0/0	0/0	0/2	0/0	0/0

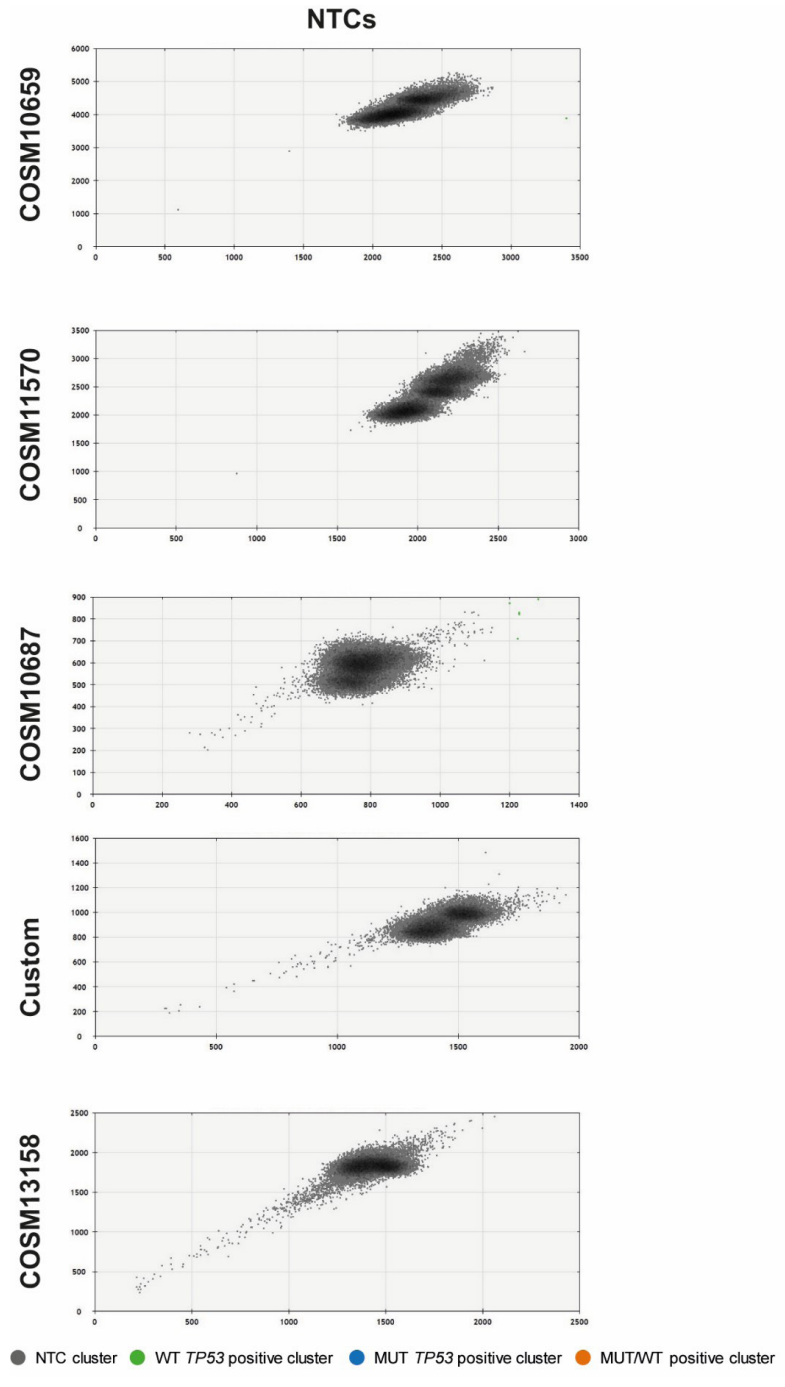
MT, mutant; WT, wildtype; VAF, variant allele frequency; FA fractional abundance



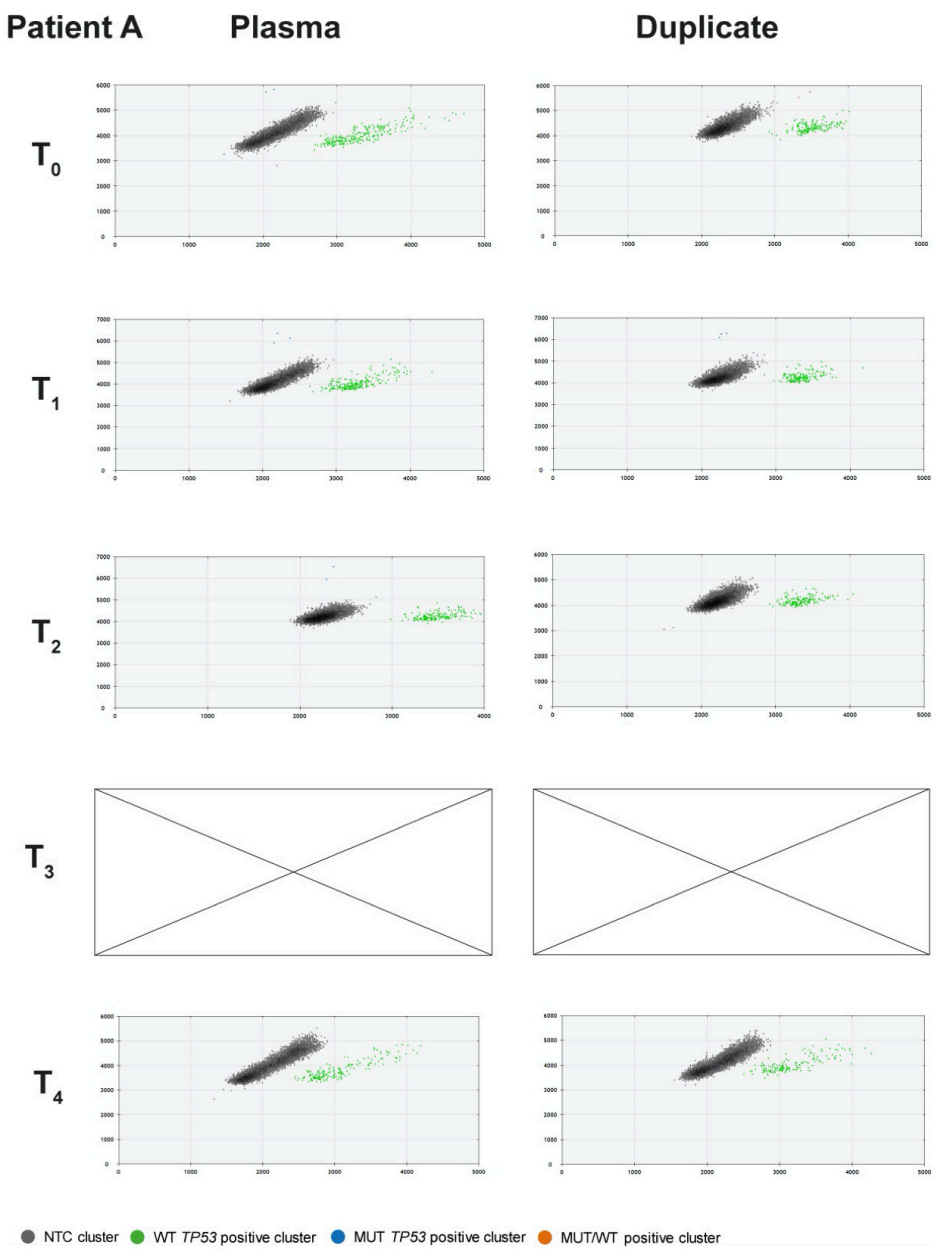
SUPPLEMENTARY FIGURE S1. Temperature gradient performed for each experimental assay in order to determine optimal annealing temperature during PCR.

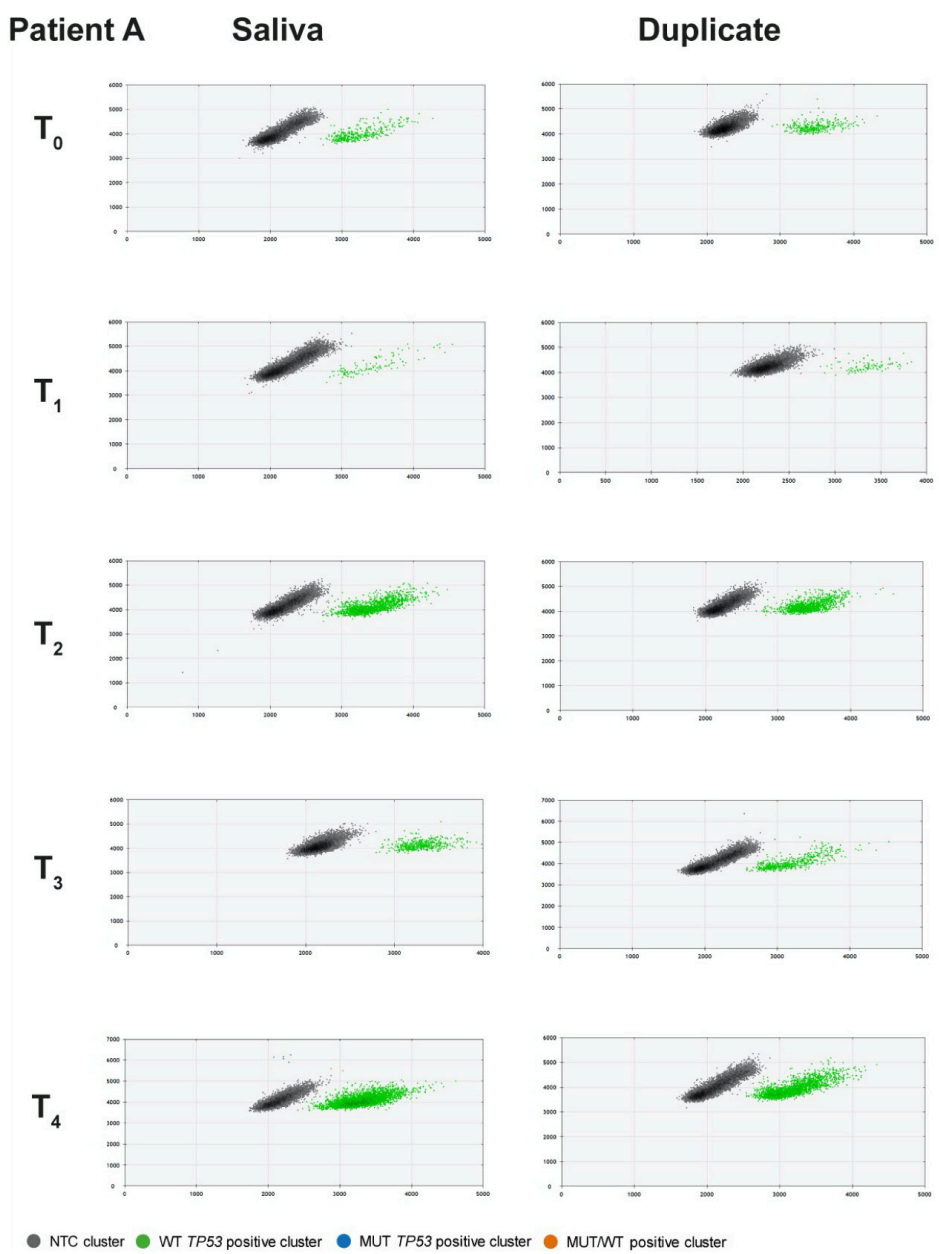


SUPPLEMENTARY FIGURE S2. Combined ddPCR results of WT-only samples in order to estimate false-positive rates for each experimental assay. Each diagram represents merged results of duplicate samples showing MT-positive droplet clusters (blue dots), negative droplet clusters (dark grey dots), and MT/WT-positive droplets (orange dots). Green dots represent WT-positive droplets, proving existence of cfDNA in the samples and satisfactory ddPCR conditions. Purple lines are manually placed thresholds for distinguishing positive and negative droplets, which were set at fluorescence values based on ddPCR results of FFPE samples.

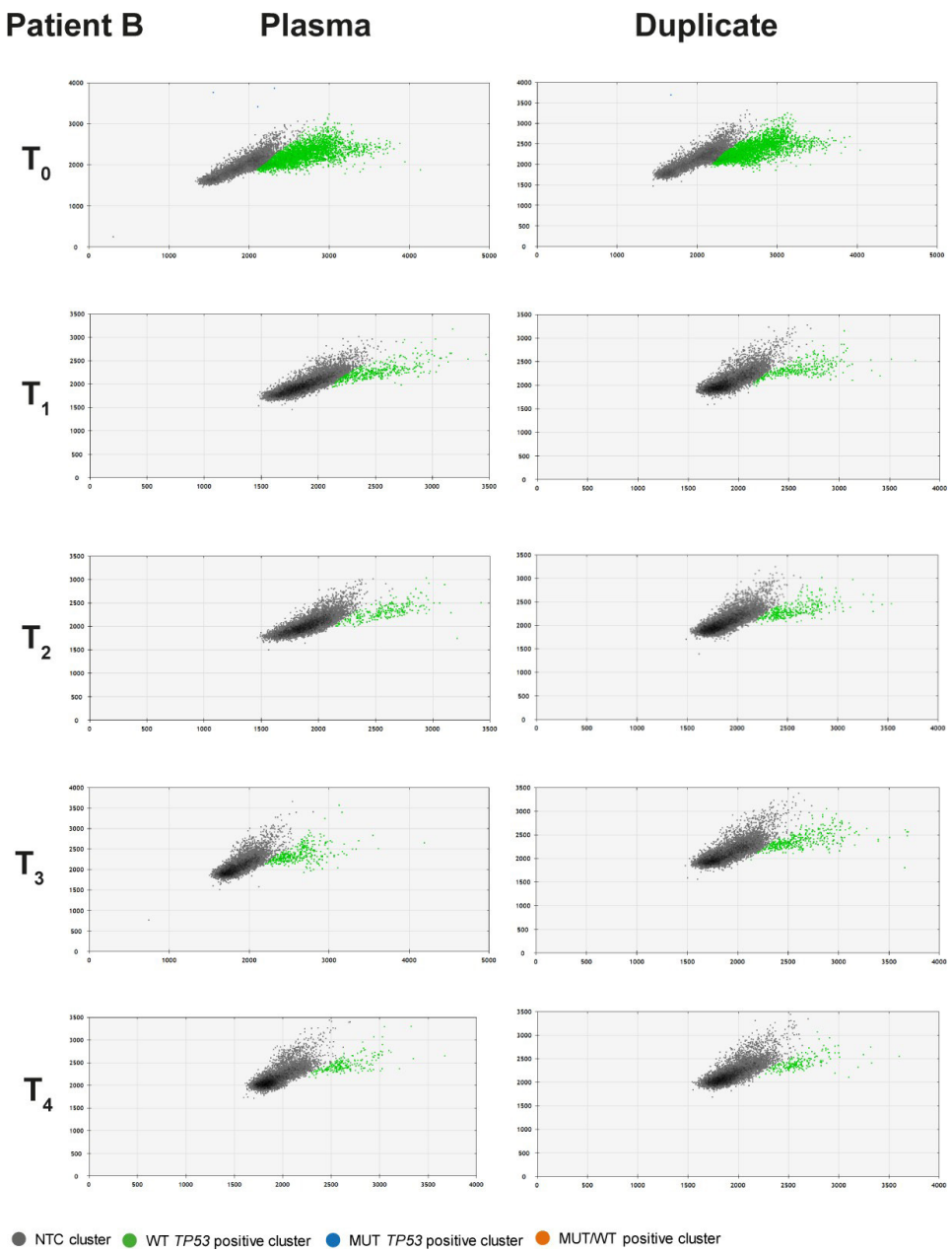


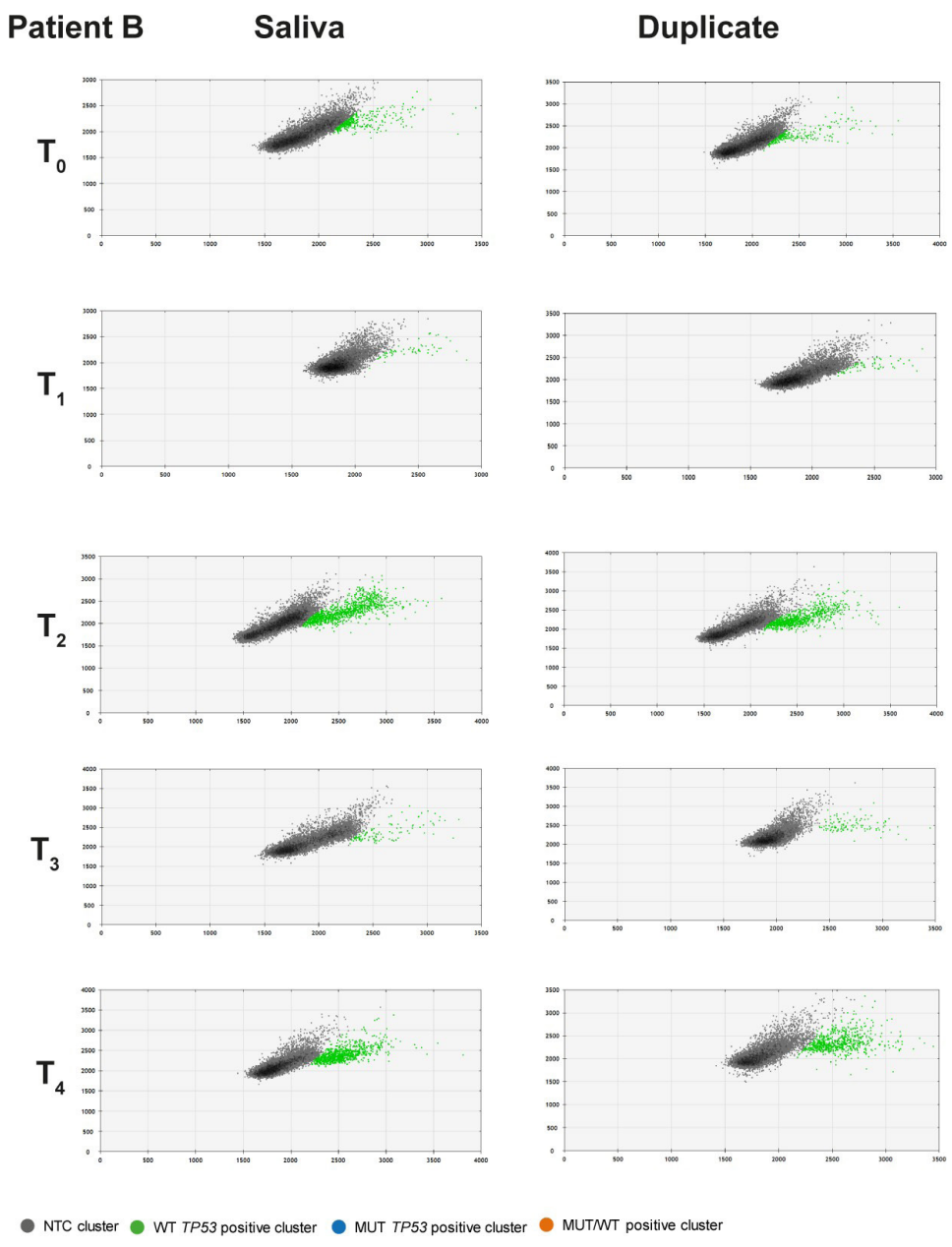
SUPPLEMENTARY FIGURE S3. Merged ddPCR results of NTC samples tested with each experimental assay for environmental contamination.



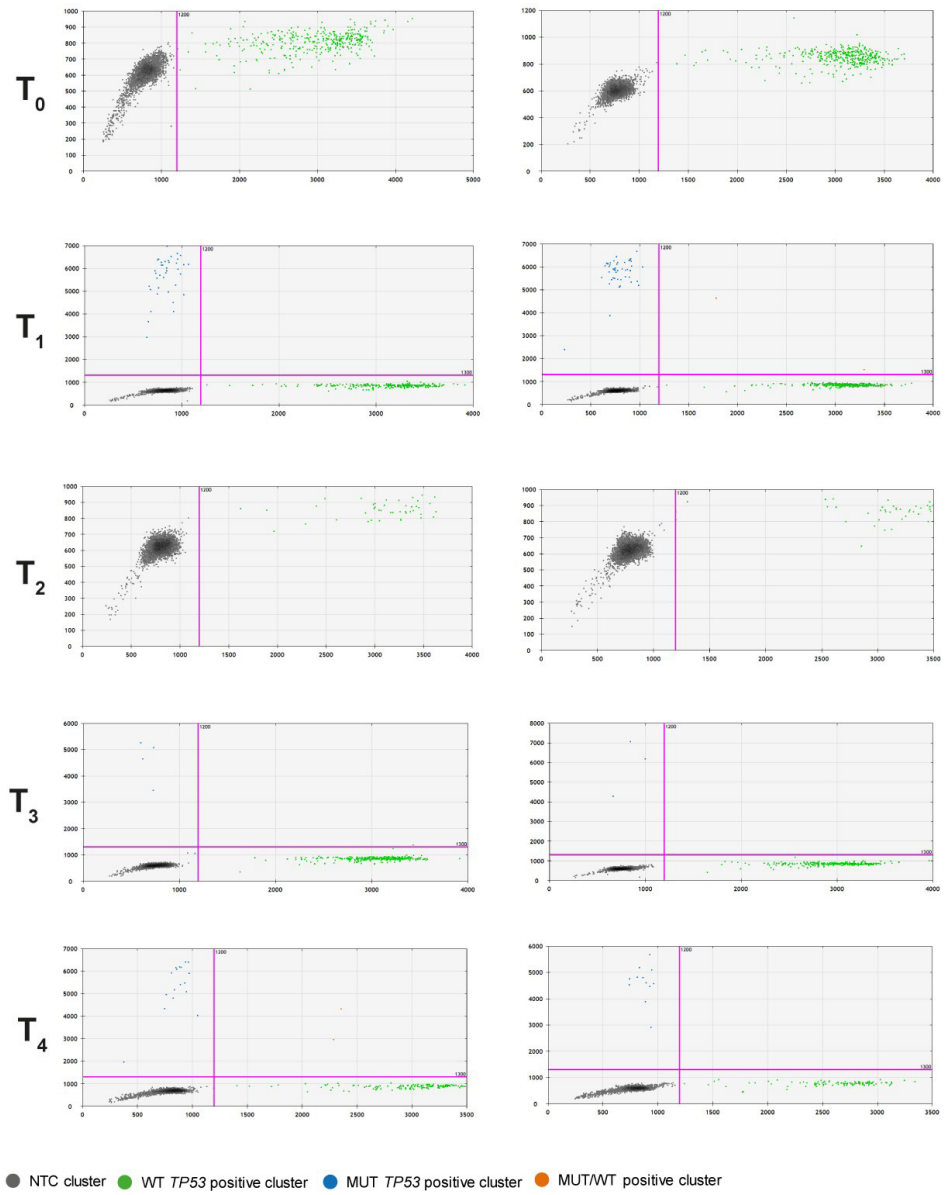


Supplementary Figures S4. DdPCR results of experimental plasma and saliva samples collected from patients A-E at T1 – T4. Note the absence of analysis results of plasma from patient A at T3 due to loss of sample.


Patient B
Plasma
Duplicate
T0
T1
T2
T3
T4
NTC cluster
WT TP53 positive cluster
MUT TP53 positive cluster
MUT/WT positive cluster

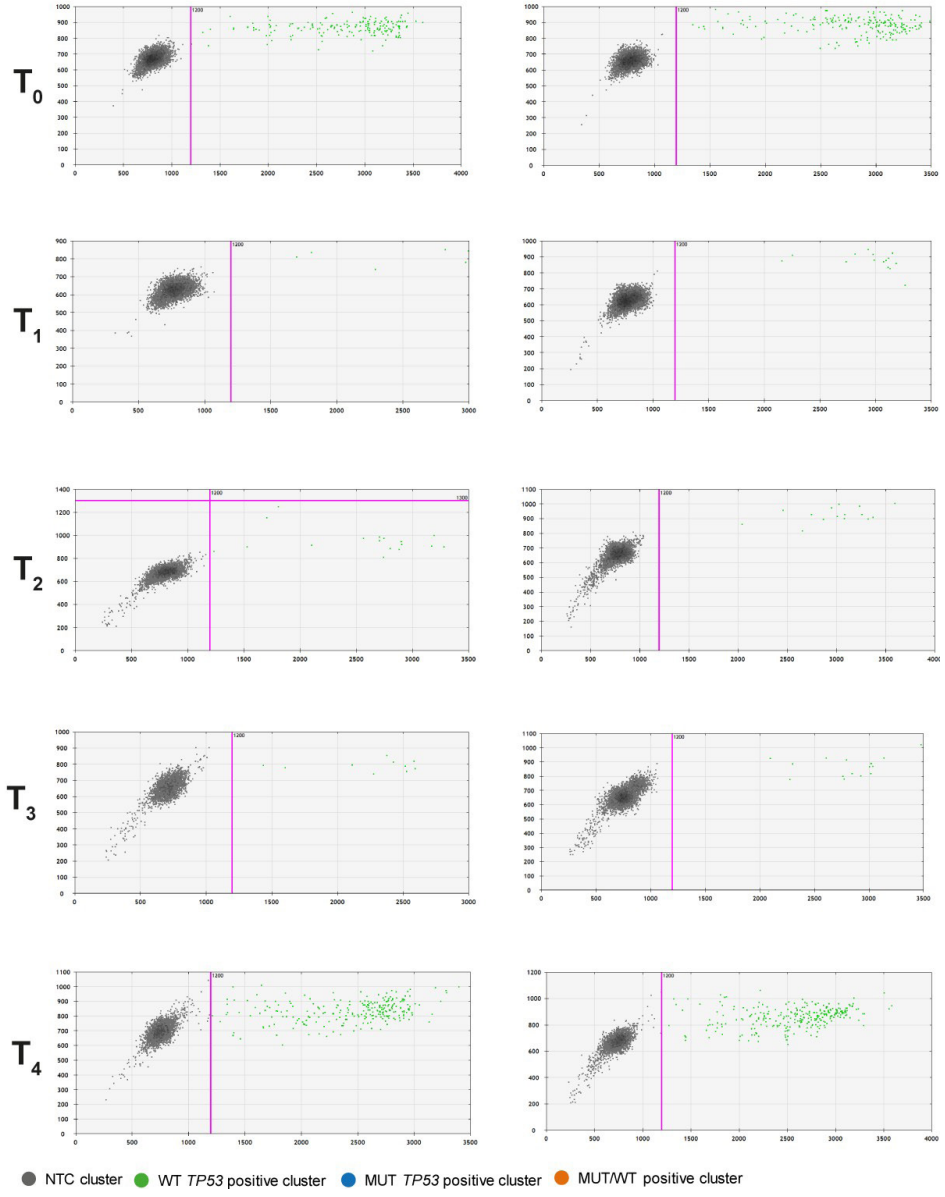

Patient B
Saliva
Duplicate
T0
T1
T2
T3
T4
NTC cluster
WT TP53 positive cluster
MUT TP53 positive cluster
MUT/WT positive cluster

Patient C **Plasma** **Duplicate**



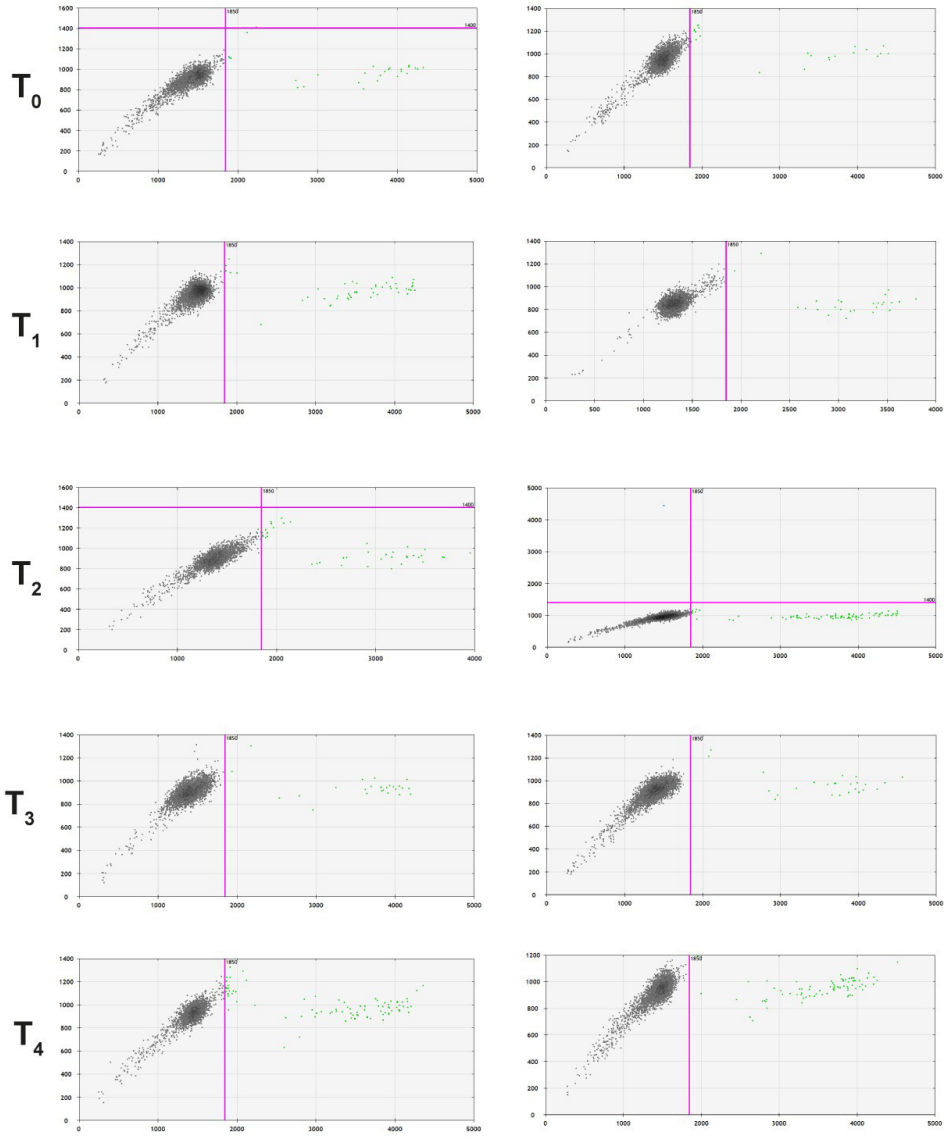
NTC cluster WT *TP53* positive cluster MUT *TP53* positive cluster MUT/WT positive cluster

Patient C **Saliva** **Duplicate**



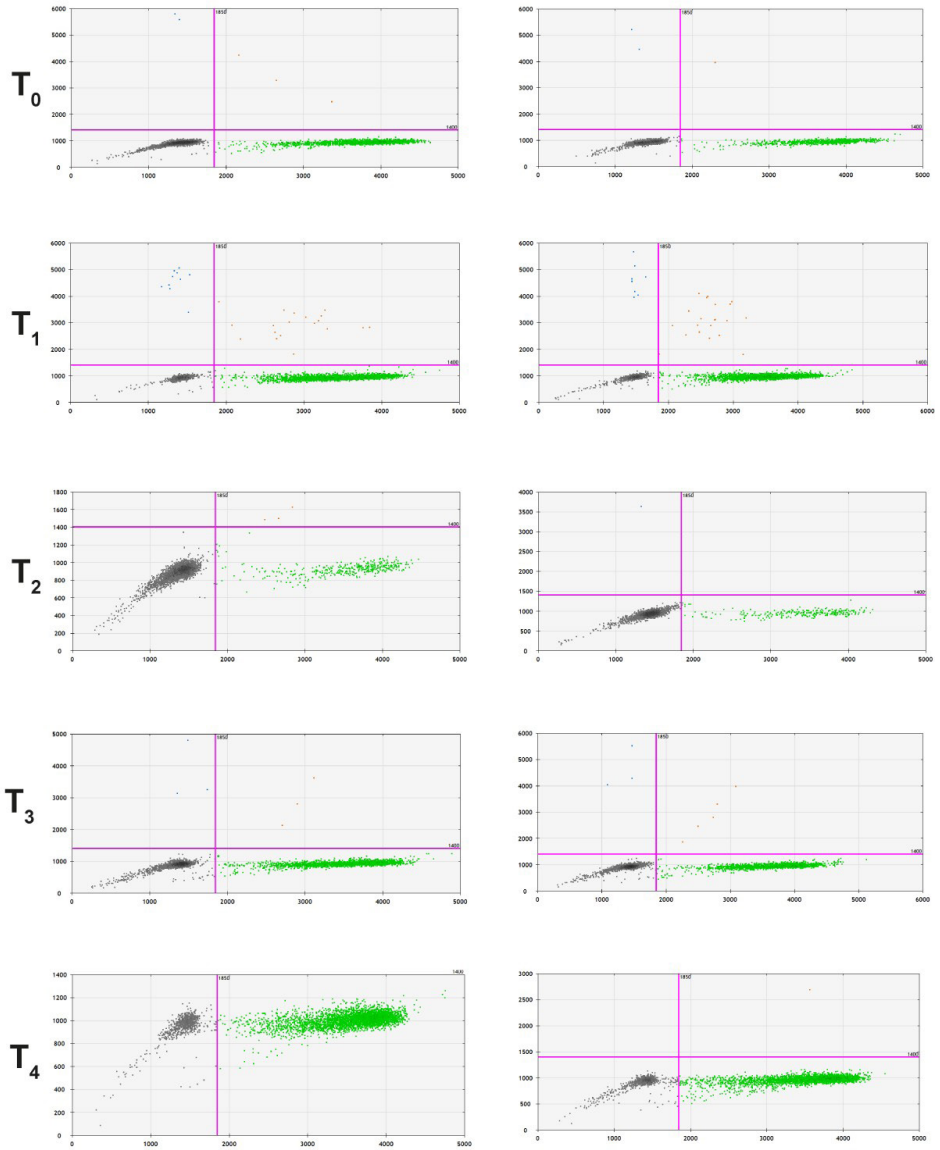
NTC cluster WT *TP53* positive cluster MUT *TP53* positive cluster MUT/WT positive cluster

Patient D **Plasma** **Duplicate**



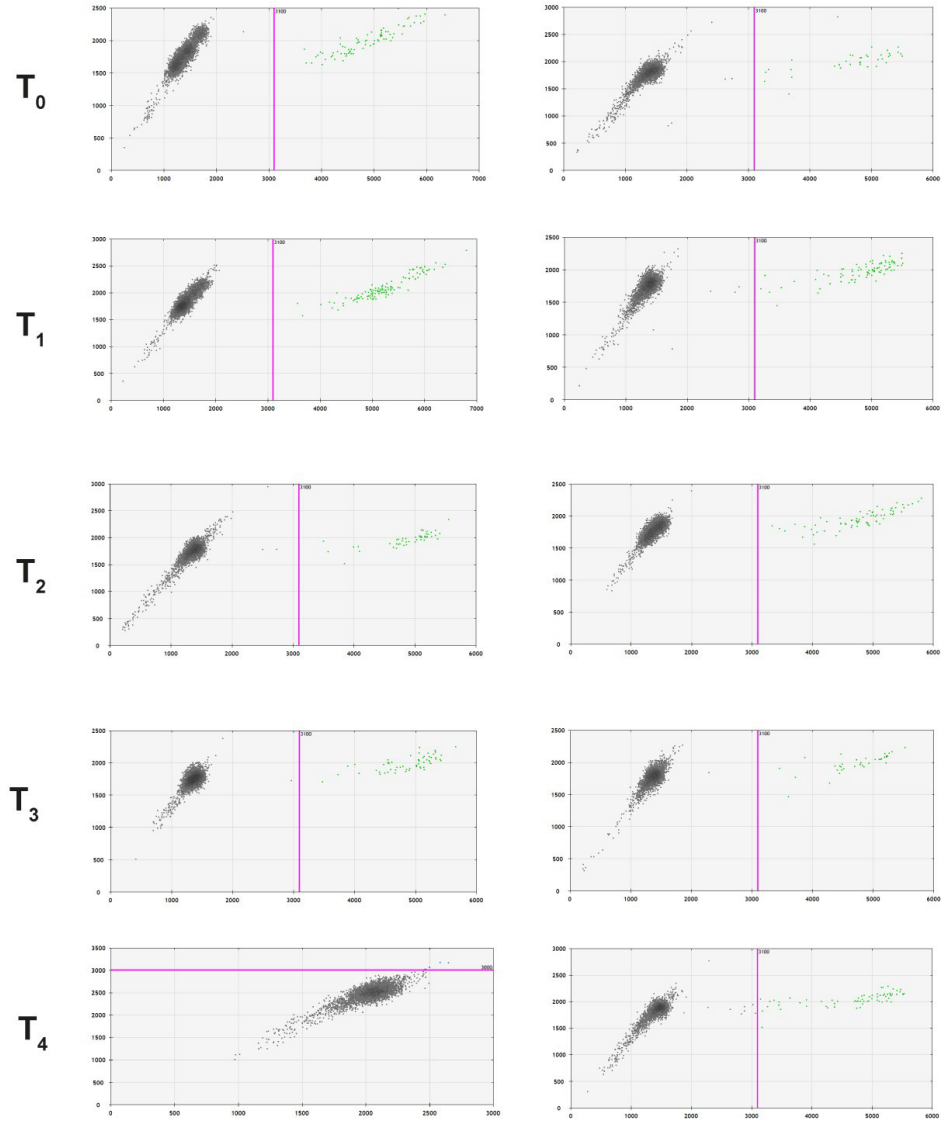
NTC cluster WT *TP53* positive cluster MUT *TP53* positive cluster MUT/WT positive cluster

Patient D **Saliva** **Duplicate**

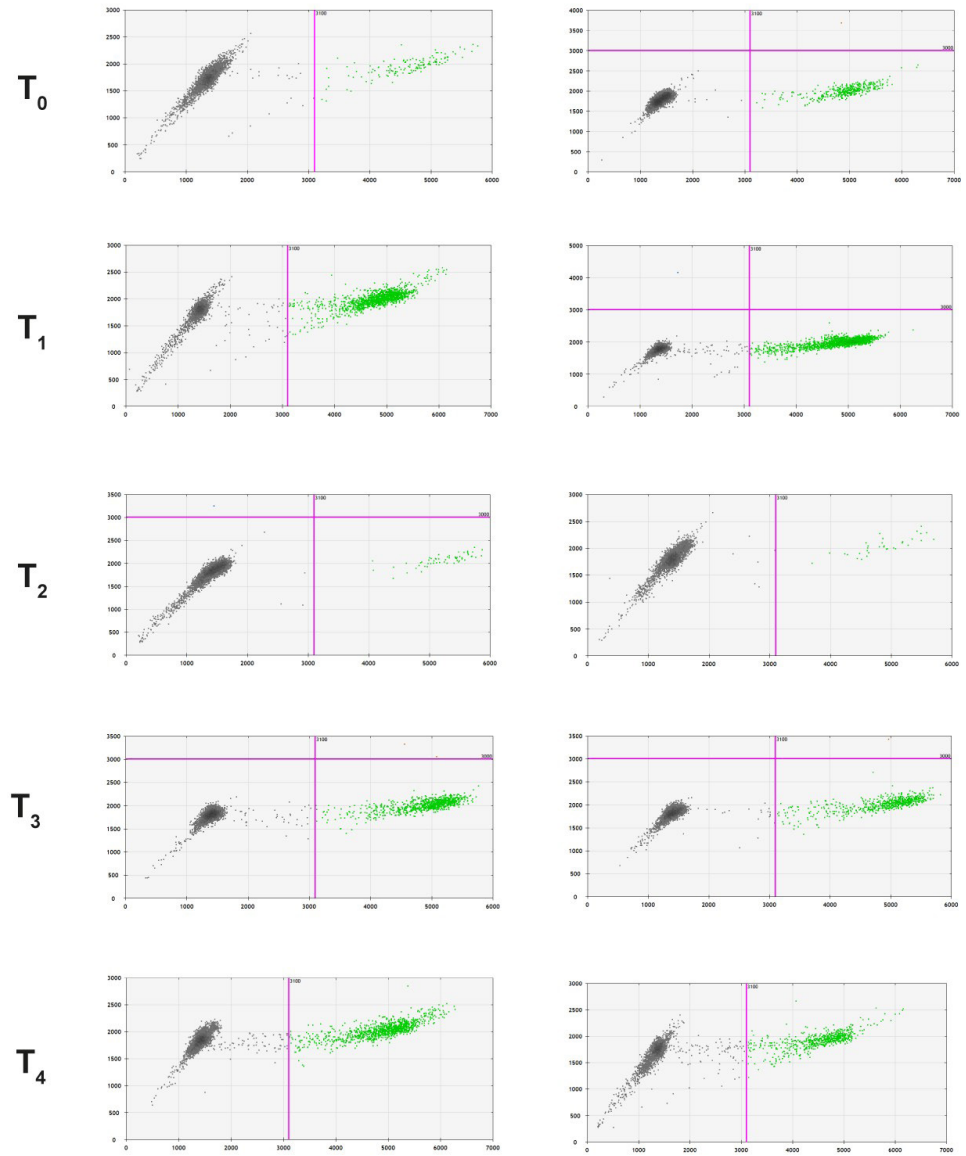


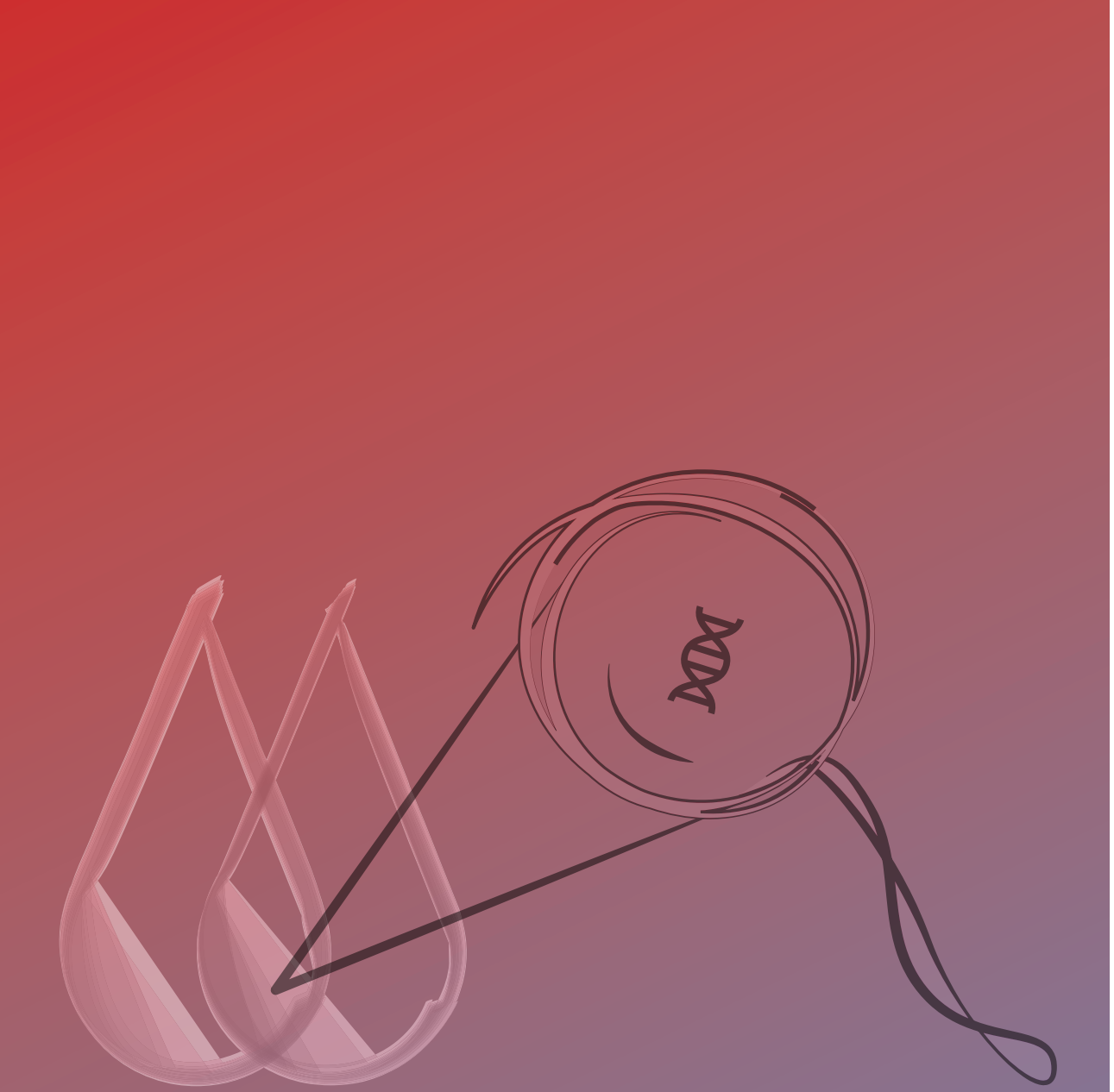
NTC cluster WT *TP53* positive cluster MUT *TP53* positive cluster MUT/WT positive cluster

Patient E **Plasma** **Duplicate**



Patient E **Saliva** **Duplicate**





7

Summarizing Discussion & Future Perspectives

SUMMARIZING DISCUSSION

Head and neck cancers remain difficult to treat due to their aggressive biological behavior and delicate functional anatomy from which they arise. This particularly applies for advanced and recurrent cases, when feasible treatment options are becoming limited due to expected poor patient survival and/or functional outcome. Salvage surgery is the treatment modality of choice in case of resectable locoregional recurrence. However, recurrent head and neck squamous cell carcinoma (HNSCC) is often detected in a late stage, rendering salvage surgery with curative intent impossible. Further, for a considerable number of patients undergoing salvage surgery with curative intent, outcomes remain poor due to a high risk of complications and morbidity [1]. Although new treatment strategies such as adjuvant immunotherapy are being developed and existing diagnostics are improving [2, 3], 5-year overall survival rates of HNSCC patients in The Netherlands have remained stable at 57%-58% over the past few decades [4, 5]. An absolute increase in incidence from 15% to 50% of prognostically favorable HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) cases has been observed in The Netherlands in the same period, even further indicating rather stagnant survival numbers of HPV-negative HNSCC cases [5- 7]. This emphasizes the need for early detection of recurrent disease in order to improve chances for a successful curative treatment plan instead of a palliative regimen. In the era of emerging precision medicine, a more personalized approach to HNSCC care is desired to pave the road for new, more accurate methods of early detection of recurrence, prognostic stratification, and therapeutic response prediction. Ideally, molecular biomarkers are being used that accurately represent tumor (epi)genetic status, and at the same time allow minimally invasive collection. Lastly, this has to be practically feasible for implementation into routine clinical practice. Therefore, the aim of this thesis was to identify potential liquid biopsy based diagnostic biomarkers, and investigate whether they are suitable for clinical implementation and use for the early detection of recurrent or residual HNSCC in order to improve the overall survival and quality of life of HNSCC patients in the future.

Previously reported results of whole-genome sequencing on HNSCC tissue already demonstrated high rates of *TP53* mutations in HPV-negative tumors [8-10]. These findings were confirmed in **Chapter 2** through analysis of clinically acquired next-generation sequencing (NGS) data of more than 200 tumor samples, also including the comparison of a subset of matched primary and recurrent or metastatic tumors. NGS was primarily based on 3 different HNSCC hotspot panels targeting 36-50 genes. Mutations in *TP53* appeared to be abundant in the vast majority of primary HPV-negative HNSCCs and their matching recurrent or metastatic tumors that were exclusively related to alcohol and/or smoking, while *PIKC3A* mutations were more often found in non-smoking/non-drinking related HPV-negative tumors. Additionally, high mutational concordance rates were found in the genetic

profiles of matched primary tumors and recurrences or metastases, of which *TP53* mutations were often synonymous in clonal outgrowths compared to mutations in other genes such as *HRAS* and *PIK3CA*. Less sensitive sequencing techniques previously demonstrated similar *TP53* mutational synonymy between primary tumors and their paired clonal outgrowths [11, 12]. In contrast to HPV-negative primary tumors, recurrent and metastatic HPV-positive tumors exhibited higher rates of *TP53* mutations than the HPV-positive index primary tumors in a previous study [13]. Current analyzed cohort contained only 4 HPV-positive primary tumor samples, of which no NGS analysis results of possible matching recurrent or metastatic tumors were present. Furthermore, both *CDKN2A* and *PIK3CA* were found to be regularly co-mutated with *TP53*. These findings suggested the use of *TP53* mutations as circulating tumor DNA (ctDNA) as potential biomarkers, which encouraged to further explore the field of liquid biopsy for head and neck cancer.

In **Chapter 3**, literature on the diagnostic and/or prognostic value of cell-free nucleic acids in blood or saliva was systematically reviewed in order to identify studies on treatment monitoring and/or prognosis. The majority of these studies showed a statistically significant differential expression of one or more miRNAs, whereas only two studies reported on a significant detectability of ctDNA (RAS-gene family) and HPV-DNA in blood associated with progression free survival [14, 15]. A relatively large number of studies was found in which various specific miRNAs turned out to be differentially expressed. However, these transcriptomic markers are often part of large, complex expression profiles consisting of multiple targets that represent gene regulation in an indirect manner. This requires far more comprehensive analysis compared to ‘simple’ detection and quantification of ctDNA. The remaining studies described very small sample sizes or yielded inconclusive results. Moreover, most research appeared to be carried out in a non-standardized fashion with regard to sample collection and storage, pre-analytical workup, and analysis technique. On the whole, this review clearly presented the lack of particularly large-scale longitudinal studies on the detection of tumor DNA in blood or saliva from HNSCC patients.

In response to the lack of published research on ctDNA analysis in HNSCC patients as highlighted in Chapter 3, in **Chapter 4** it was investigated whether ultrasensitive droplet digital PCR (ddPCR) can be used for ctDNA analysis of plasma; DNA was isolated from archived formalin fixed paraffin embedded (FFPE) primary tumor biopsy samples from 6 patients diagnosed with stage II-IV, *TP53* mutant-positive, HPV-negative HNSCC. Subsequently, cfDNA was isolated from banked, frozen plasma samples of these patients. The samples were collected at the time of diagnosis. ddPCR analysis was performed on all plasma samples, using the *TP53* mutations as template for tumor specific assays. In all plasma samples, ctDNA could be detected down to fractional abundances of 0.01% within (wild-type) cfDNA backgrounds, confirming the technical feasibility of ctDNA detection in plasma samples from HNSCC patients using ddPCR.

To further improve sample workup for ctDNA analysis, optimal laboratory parameters and protocols were investigated in **Chapter 5**. In order to do this, different methods and protocols for sample collection, storage, centrifugation, and DNA isolation and quantification were compared using ddPCR analysis of plasma samples from healthy individuals and lung cancer patients. Whole-blood collected in silicone coated tubes for serum analysis yielded higher concentrations of isolated DNA than whole-blood collected in tubes containing heparin, citrate, and ethylenediaminetetraacetic acid (EDTA). However, serum samples were likely contaminated with higher amounts of genomic DNA, since serum is prone to clotting induced cell lysis [16]. Furthermore, cfDNA concentrations in blood samples collected in EDTA tubes remained stable for at least 24 hours of storage at room temperature, and two-step centrifugation of samples was preferred prior to cfDNA isolation in order to remove the cellular component optimally. Different commercially available cfDNA isolation kits such as QIAamp and MagNAPure performed equally well, but Zymo Quick yielded highest DNA concentrations in plasma. For quantity control following DNA isolation, Qubit proved more accurate than Nanodrop.

In line of Chapters 4 and 5, an observational pilot study was started, for which the aim was to establish a streamlined clinical workflow for the collection and analysis of blood and saliva samples from HNSCC patients during definitive (chemo)radiotherapy. In **Chapter 6** preliminary results of five included patients of this ongoing study were shown. Various changing patterns in ctDNA concentrations were observed in these patients during the course of treatment. These patterns differed among patients and between sample types. Although no clear trend was recognizable in analysis results, first important steps have been taken towards the implementation of practical research protocols into clinical practice for a streamlined workflow including study related acts. This also facilitates the initiation of new projects on the subject of liquid biopsy. Complementing study results are desired and will hopefully contribute to new insights on the use of liquid biopsies in HNSCC patients, and provide ground for further research.

In summary, this thesis has contributed to the fast growing field of liquid biopsy in HNSCC patients by exploring a wide variety of (pre)clinical aspects, and thereby paved the road for further, more focused research on ctDNA analysis in HNSCC patients. Although several of these aspects remain unclear, this thesis provides a lead for further investigating the use of liquid biopsies for personalized head and neck cancer management in order to improve patient disease outcome and quality of life ultimately.

FUTURE PERSPECTIVES

Current status of liquid biopsy in head and neck cancer

Biomarkers can be categorized as diagnostic, prognostic, or predictive [17]. Diagnostic biomarkers are used predominantly for early disease detection and treatment monitoring, while prognostic and predictive biomarkers are both associated with outcomes, regardless of treatment and to a specific treatment respectively. To date, no liquid biopsy based biomarkers are used in daily clinical practice for HNSCC patients yet [18]. Research on liquid biopsy for disease monitoring, stratification and prognostication is progressing mostly due to its investigative possibilities for patient-friendly observational study designs, which allow for the retrieval of large amounts of data in a relatively short period of time through serial sampling of body fluids. Research on treatment response prediction has to follow an entirely different path, as determining the clinical significance of a certain predictive biomarker not only requires analysis of its detectability in liquid biopsies, but also its predictive value for a targeted treatment. This also incorporates co-development of a therapeutic product under preclinical conditions before being applied in a clinical phase for assessment of biomarker-associated outcome [19]. Nonetheless, several downstream targets affecting the *PI3K/AKT/MTOR* pathway such as *PIK3CA* [20-23] and *EFGR* [24-27] are currently under preclinical investigation and *TP53* mutations for novel treatments in oral cancer patients are being explored [28].

Health care costs are another important factor that has to be taken into account with the (increase in) ageing population. Even though elderly patients receive significantly more often no or less (intense) treatment when compared to patients younger than 65 years, absolute numbers of elderly patients receiving treatment will rise as the number of head and neck cancer increased by almost 45% between 2000 and 2014 in the 65-plus category and by 70% in the population beyond 80 years of age [29]. Health care utilization and associated costs of treatment of HNSCC patients with recurrent or metastatic disease are fairly high in The Netherlands, while survival is limited [30]. Molecular cancer diagnostics have the potential to rapidly evolve into a more accurate, less harmful and more cost-effective method for early detection of recurrent HNSCC than, for instance, conventional imaging diagnostics due to its more tumor specific approach and minimally invasive character.

Challenges of liquid biopsy in head and neck cancer

One of the major challenges for the clinical application of liquid biopsy for HNSCC is intratumor heterogeneity. Random or therapy-induced somatic mutations that accumulate within tumor cells cause the emergence of subclonal populations within a tumor [31]. Although *TP53* mutations appear to be inherited quite consistently throughout subclonal evolution from primary tumor to clonal outgrowth, this applies less for somatic mutations in

other genes as shown in chapter 3 and by others [12]. Tumor DNA being shed into blood and saliva by genetically distinct subclones within a primary tumor or in the form of locoregional recurrence or distant metastasis thus might not reflect actual tumor presence or burden accurately. And while single target analysis of *TP53* mutations using ddPCR appears to be conceptually and technically promising, it is limited in case of absence of these mutations as preferred targets or an unknown primary tumor mutational profile. Genetic heterogeneity further causes spatiotemporal divergence in tumor phenotype such as morphological appearance, invasive growth behavior, therapy-resistance, and vascularization from its surrounding microenvironment [32, 33]. These factors determine rate and degree of tumor cell necrosis and apoptosis, and direct access to blood vessels or saliva. This would conceivably compromise tumor DNA detection in body fluids by affecting ctDNA kinetics [34].

Posttreatment minimal residual disease (MRD) may be another possible challenge for future liquid biopsy development [35]. Although not fully clarified yet, it is suggested that clinically and radiologically occult tumor cells, also known as micrometastases, leave the primary tumor and disseminate lymphatically to regional lymph nodes, or migrate to the bloodstream as circulating tumor cells (CTC) to distant sites where they thrive as dormant disseminated tumor cells, and appear to be responsible for the development of distant metastases when they start to proliferate [36]. Therefore, timely detection of MRD is desired in order to diagnose HNSCC patients with pending metastatic disease, before distant organs are being seeded. However, detection of MRD through liquid biopsy remains challenging because of very low CTC counts and ctDNA levels, and due to current limitations of individualized primary tumor sequencing and biomarker detection protocols [37].

Similar to MRD, the phenomenon of field cancerization may further complicate development of liquid biopsy based biomarker analysis. Field cancerization is the biological concept of genetic evolution of normal mucosal epithelium into a premalignant field by dysplastic changes due to prolonged local exposure to carcinogens. Multiple genetically related tumors, or 'second field tumors', can arise newly from premalignant fields at surgical resection margins, developing into a true local recurrence after primary treatment [38]. This hampers posttreatment disease monitoring, as liquid biopsy does not reflect spatiotemporal tumor evolution; if primary tumor mutations in ctDNA are targeted to track clinically undetectable MRD, co-occurrence of a second field tumor shedding genetically related ctDNA may negatively affect diagnostic accuracy by obscuring MRD presence. In turn, this would leave the clinician questioning whether being confronted by metastatic disease from a previous primary tumor or by a locally developing second tumor in the affected field. This emphasizes the importance of a multimodality approach using clinical, radiological, and (molecular) pathological expertise.

Meanwhile, existing ctDNA detection techniques such as NGS and ddPCR are under constant technical development and adaptation in order to increase diagnostic power

and possibilities (e.g. ultrasensitive multiplexing capabilities for accurate high-throughput multiple target analysis) and reduce costs [39, 40], whereas newer techniques such as single-molecule (real-time) sequencing address the above mentioned challenges for liquid biopsy development through different bioanalytical approaches [41].

Future research on liquid biopsy

Liquid biopsy in oncology is currently of high interest for various cancers, given the large amount of studies emerging on this subject. Therefore, different oncological fields should mutually benefit from future research results in the first place. In general, translational biomarker research entails a complex of progressing stages depending on the application and purpose for which it is conducted. Standardization is pivotal for establishing large-scale lines of research in order to reliably assess clinical validity and utility by connecting bench with bedside. In this aspect, small steps have already been made in chapters 5 and 6. However, similar studies need to be performed in multiple laboratories from different institutes in order to reach consensus on research outcomes more readily.

Similarly as ctDNA, CTCs could be used to genetically analyze tumor DNA for targeted treatment strategies and to detect a recurrence or progression of disease after initial therapy. The main principle of this technique relies on the identification of CTCs by enrichment of cells expressing epithelial cell adhesion molecules followed by immunofluorescent staining using different markers. In a pooled analysis of 1944 patients with metastatic breast cancer, an independent prognostic effect of CTC enumeration on progression-free survival and overall survival was confirmed [42]. All patients were analyzed at the start of a new therapy. Patients with a CTC count of 5 per 7.5 ml or higher at the start of treatment were associated with a decreased progression-free survival and overall survival ($p<0.0001$), compared to patients with a CTC count of less than 5 per 7.5 ml. Another field of interest in current research is CTC characterization. Several studies investigated protein expression, RNA expression and DNA aberrations in CTCs to help guide drug therapy in different cancer types. Especially HER2 protein expression in breast cancer patients and AR signaling in castration resistant prostate cancer are subjects of interest in current studies [43]. As cancers of the salivary glands have characteristics comparable to breast and prostate cancers, the application of CTC analysis in these cases can be expected in the near future [44, 45]. There are however, certain issues pertaining to the use of CTCs before translating into the therapeutic arena, particularly with regard to detection and characterization of the genetic alterations, because CTC concentration in blood circulation is very low. Current estimations on CTC concentration is one tumor cell per one billion of normal blood cells. A sensitive way to detect, isolate and differentiate CTCs thereby remains challenging. The detection of CTCs is also dependent on the timing of blood sampling. In HNSCC studies, the portion of patients with positive CTC levels varies from 6.5 to 87.5% [46-49].

Focusing on the use of ctDNA, bioanalytical studies on the biomechanism and -dynamics of ctDNA are needed, in which simultaneous imaging should be implemented to be able to correlate radiological tumor burden with DNA levels. Also, new detection methods other than ddPCR are emerging, eventually requiring its validation in clinical sample sets by comparing these with existing methods. Therefore, the use of different ultrasensitive sequencing and/or digital PCR platforms in parallel for the analysis of clinical samples will generate multipurpose data on clinical utility, technical validation, and comparison of different diagnostic tests. Clinically, carrying out pre- and posttreatment liquid biopsies in surgically treated oral cancer patients that received postoperative (chemo)radiation is of interest to investigate possibilities for treatment response monitoring on recurrent disease during 2-year follow-up by comparison with standard of care (i.e. clinical evaluation supported by radiology). The main goal of the study described in Chapter 6 is to explore the feasibility of *TP53* mutation detection in blood and saliva from HNSCC patients in a clinical setting. Its protocol is recently extended by adding posttreatment liquid biopsies to carry out at 3 months during follow-up and at evidence of clinical recurrence. Also, the inclusion of HPV-positive HNSCC patients is planned, for which cell-free HPV-DNA will be used as biomarker. Additionally, single-molecule real-time sequencing analysis of samples will be performed in parallel with ddPCR in the future.

Multicenter (nationwide) prospective studies are preferred in order to clarify cell-free tumor DNA kinetics in blood and saliva more comprehensively, and to be able to validate promising results of small-scale research. Ultimately, the focus should be on integrating clinical and radiological data into biomarker analysis as a prelude towards personalized head and neck cancer care.

REFERENCES

1. Hamoir M, Schmitz S, Suarez C, Strojan P, Hutcheson KA, Rodrigo JP, et al. The Current Role of Salvage Surgery in Recurrent Head and Neck Squamous Cell Carcinoma. Cancers. 2018;10.
2. Kareemaghay S, Tavassoli M. Clinical immunotherapeutic approaches for the treatment of head and neck cancer. International journal of oral and maxillofacial surgery. 2019;48:419-36.
3. Martens RM, Noij DP, Koopman T, Zwezerijnen B, Heymans M, de Jong MC, et al. Predictive value of quantitative diffusion-weighted imaging and 18-F-FDG-PET in head and neck squamous cell carcinoma treated by (chemo) radiotherapy. Eur J Radiol. 2019;113:39-50.
4. Marur S, Forastiere AA. Head and Neck Squamous Cell Carcinoma: Update on Epidemiology, Diagnosis, and Treatment. Mayo Clinic proceedings. 2016;91:386-96.
5. Braakhuis BJ, Leemans CR, Visser O. Incidence and survival trends of head and neck squamous cell carcinoma in the Netherlands between 1989 and 2011. Oral oncology. 2014;50:670-5.
6. van Monsjou HS, Schaapveld M, van den Brekel MW, Balm AJ. The epidemiology of head and neck squamous cell carcinoma in The Netherlands during the era of HPV- related oropharyngeal squamous cell carcinoma. Is there really evidence for a change? Oral oncology. 2015;51:901-7.
7. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. The New England journal of medicine. 2010;363:24-35.
8. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333:1154-7.
9. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333:1157-60.
10. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517:576-82.
11. Tjebbes GWA, Van der Straat FGJL, Tilanus MGJ, Hordijk GJ, Slootweg PJ. p53 tumor suppressor gene as a clonal marker in head and neck squamous cell carcinoma: p53 mutations in primary tumor and matched lymph node metastases. Oral oncology. 1999;35:384-9.
12. Hedberg ML, Goh G, Chiosea SI, Bauman JE, Freilino ML, Zeng Y, et al. Genetic landscape of metastatic and recurrent head and neck squamous cell carcinoma. The Journal of clinical investigation. 2016;126:169-80.
13. Morris LG, Chandramohan R, West L, Zehir A, Chakravarty D, Pfister DG, et al. The Molecular Landscape of Recurrent and Metastatic Head and Neck Cancers: Insights From a Precision Oncology Sequencing Platform. JAMA Oncol. 2016.
14. Braig F, Voigtlaender M, Schieferdecker A, Busch C-J, Laban S, Grob T, 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;7:42988-95.
15. Ahn SM, Chan JY, Zhang Z, Wang H, Khan Z, Bishop JA, 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;140:846-54.
16. Board RE, Williams VS, Knight L, Shaw J, Greystoke A, Ranson M, et al. Isolation and extraction of circulating

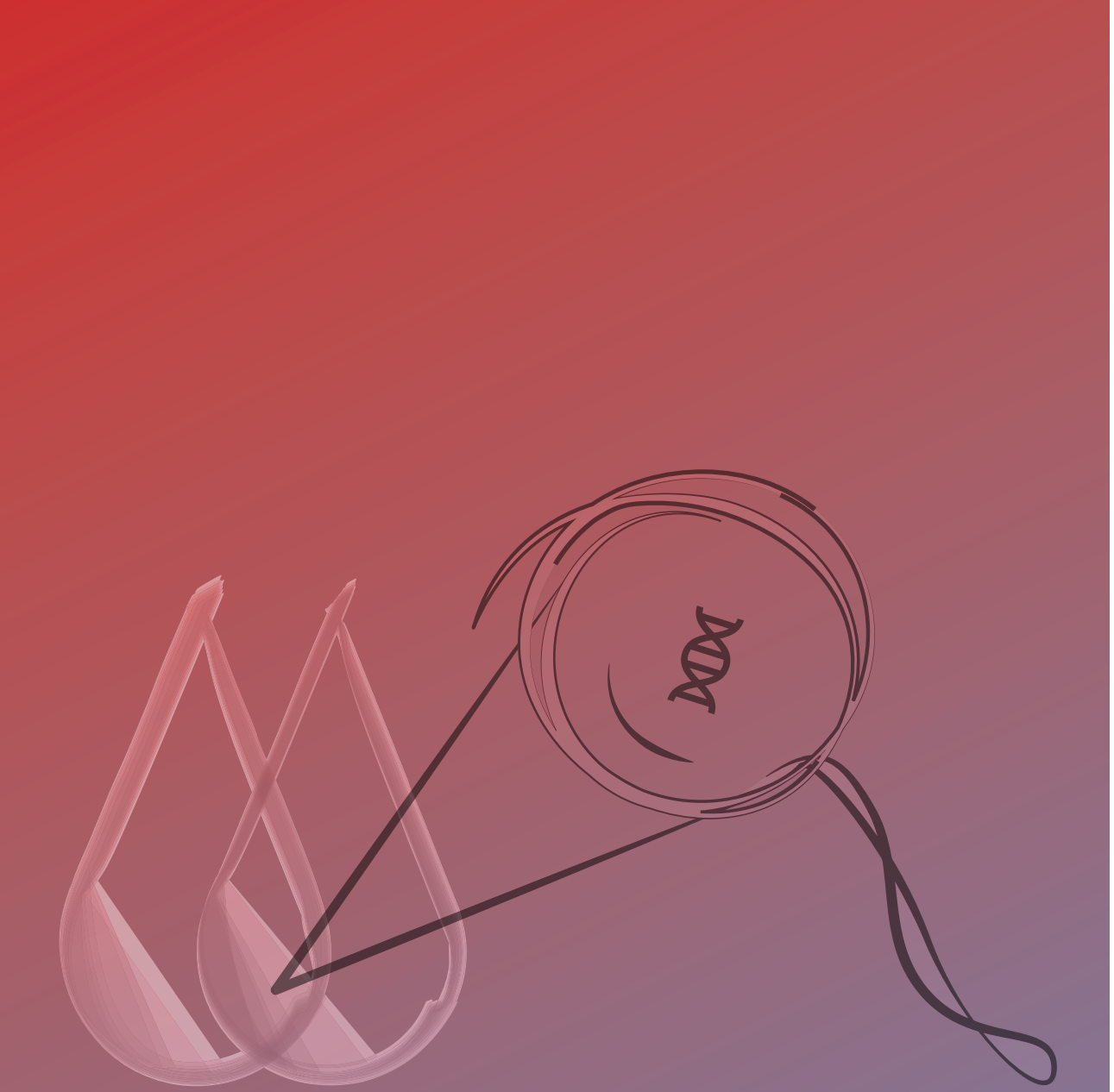
tumor DNA from patients with small cell lung cancer. Ann N Y Acad Sci. 2008;1137:98-107.

17. Kang H, Kiess A, Chung CH. Emerging biomarkers in head and neck cancer in the era of genomics. Nature reviews Clinical oncology. 2015;12:11-26.
18. Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2018;36:1631-41.
19. Shimazawa R, Ikeda M. Drug-diagnostic co-development: challenges and issues. Expert review of molecular diagnostics. 2016;16:187-204.
20. Lui VW, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. Cancer discovery. 2013;3:761-9.
21. Wang Z, Martin D, Molinolo AA, Patel V, Iglesias-Bartolome R, Degese MS, et al. mTOR co-targeting in cetuximab resistance in head and neck cancers harboring PIK3CA and RAS mutations. Journal of the National Cancer Institute. 2014;106.
22. Hedberg ML, Peyser ND, Bauman JE, Gooding WE, Li H, Bhola NE, et al. Use of nonsteroidal anti-inflammatory drugs predicts improved patient survival for PIK3CA- altered head and neck cancer. The Journal of experimental medicine. 2019;216:419- 27.
23. 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.
24. Bozec A, Etienne-Grimaldi MC, Fischel JL, Sudaka A, Toussan N, Formento P, et al. The mTOR-targeting drug temsirolimus enhances the growth-inhibiting effects of the cetuximab-bevacizumab-irradiation combination on head and neck cancer xenografts. Oral oncology. 2011;47:340-4.
25. Bozec A, Ebran N, Radosevic-Robin N, Sudaka A, Monteverde M, Toussan N, et al. Combination of mTOR and EGFR targeting in an orthotopic xenograft model of head and neck cancer. Laryngoscope. 2016;126:E156-63.
26. Lattanzio L, Milano G, Monteverde M, Tonissi F, Vivenza D, Merlano M, et al. Schedule-dependent interaction between temsirolimus and cetuximab in head and neck cancer: a preclinical study. Anti-cancer drugs. 2016;27:533-9.
27. Bozec A, Ebran N, Radosevic-Robin N, Chamorey E, Yahia HB, Marcie S, et al. Combination of phosphotidylinositol-3-kinase targeting with cetuximab and irradiation: A preclinical study on an orthotopic xenograft model of head and neck cancer. Head & neck. 2017;39:151-9.
28. Lindemann A, Takahashi H, Patel AA, Osman AA, Myers JN. Targeting the DNA Damage Response in OSCC with TP53 Mutations. Journal of dental research. 2018;97:635-44.
29. Halmos GB, Bras L, Siesling S, van der Laan B, Langendijk JA, van Dijk BAC. Age-specific incidence and treatment patterns of head and neck cancer in the Netherlands-A cohort study. Clin Otolaryngol. 2018;43:317-24.
30. van der Linden N, Buter J, Pescott CP, Lalisang RI, de Boer JP, de Graeff A, et al. Treatments and costs for recurrent and/or metastatic squamous cell carcinoma of the head and neck in the Netherlands. Eur Arch Otorhinolaryngol. 2016;273:455-64.
31. Zhang XC, Xu C, Mitchell RM, Zhang B, Zhao D, Li Y, et al. Tumor evolution and intratumor heterogeneity of an oropharyngeal squamous cell carcinoma revealed by whole-genome sequencing. Neoplasia. 2013;15:1371-8.
32. Puram SV, Tirosh I, Parikh AS, Patel AP, Yizhak K, Gillespie S, et al. Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer. Cell. 2017;171:1611-24 e24.
33. Kim C, Suh JY, Heo C, Lee CK, Shim WH, Park BW, et al. Spatiotemporal heterogeneity of tumor vasculature during tumor growth and antiangiogenic treatment: MRI assessment using permeability and blood volume parameters. Cancer medicine. 2018;7:3921-34.
34. Muhanna N, Di Grappa MA, Chan HHL, Khan T, Jin CS, Zheng Y, et al. Cell-Free DNA Kinetics in a Pre-Clinical Model of Head and Neck Cancer. Scientific reports. 2017;7:16723.
35. Sproll C, Fluegen G, Stoecklein NH. Minimal Residual Disease in Head and Neck Cancer and Esophageal Cancer. Adv Exp Med Biol. 2018;1100:55-82.
36. Grobe A, Blessmann M, Hanken H, Friedrich RE, Schon G, Wikner J, 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. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014;20:425-33.
37. Pantel K, Alix-Panabieres C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. Nature reviews Clinical oncology. 2019.
38. Braakhuis BJ, Leemans CR, Brakenhoff RH. A genetic progression model of oral cancer: current evidence and clinical implications. Journal of oral pathology & medicine: official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2004;33:317-22.
39. Horak P, Frohling S, Glimm H. Integrating next-generation sequencing into clinical oncology: strategies, promises and pitfalls. ESMO Open. 2016;1:e000094.
40. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet. 2019;20:71-88.
41. Ameur A, Kloosterman WP, Hestand MS. Single-Molecule Sequencing: Towards Clinical Applications. Trends in Biotechnology. 2019;37:72-85.
42. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. The Lancet Oncology. 2014;15:406-14.
43. Jiang Y, Palma JF, Agus DB, Wang Y, Gross ME. Detection of androgen receptor mutations in circulating tumor cells in castration-resistant prostate cancer. Clin Chem. 2010;56:1492-5.
44. Szewczyk M, Marszalek A, Sygut J, Golusinski P, Golusinski W. Prognostic markers in salivary gland cancer and their impact on survival. Head & neck. 2019.
45. Kong J, Luo Y, Jin D, An F, Zhang W, Liu L, et al. A novel microfluidic model can mimic organ-specific metastasis of circulating tumor cells. Oncotarget. 2016;7:78421- 32.
46. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nature reviews Cancer. 2008;8:329-40.
47. Ignatiadis M, Dawson SJ. Circulating tumor cells and circulating tumor DNA for precision medicine: dream or reality? Annals of oncology : official journal of the European Society for Medical Oncology / ESMO.

2014;25:2304-13.

48. Mockelmann N, Laban S, Pantel K, Knecht R. Circulating tumor cells in head and neck cancer: clinical impact in diagnosis and follow-up. Eur Arch Otorhinolaryngol. 2014;271:15-21.

49. Pantel K, Speicher MR. The biology of circulating tumor cells. Oncogene. 2015.



A

SUMMARY IN DUTCH

Nederlandse samenvatting

Maligne hoofd-halstumoren kunnen moeilijk te behandelen zijn vanwege hun agressieve biologische karakter en de precaire functionele anatomie waarin deze hun oorsprong vinden. Dit is met name het geval bij vergevorderde en terugkerende ziekte. Vergevorderde hoofd-halsplaveiselcelcarcinomen worden doorgaans behandeld met een combinatiebehandeling van chirurgie en radiotherapie met of zonder chemotherapie of radiotherapie met of zonder chemotherapie met zogenaamde salvage chirurgie achter de hand bij residu of recidief tumor. In opzet curatief opereren is de behandeloptie van eerste keus in geval van een resecteerbaar locoregionaal recidief na primaire radiotherapie met of zonder chemotherapie. Recidieven van hoofd-halsplaveiselcelcarcinomen worden echter vaak pas in een laat stadium ontdekt, met als gevolg dat een genezende operatie niet altijd meer mogelijk is. Bovendien zijn de resultaten van dergelijke behandeling vaak teleurstellend, vanwege een hoog risico op complicaties en morbiditeit. Alhoewel nieuwe behandelmethoden worden ontwikkeld zoals adjuvante immunotherapie en daarnaast reeds bestaande diagnostiek steeds verder verbetert, is de 5-jaarsoverlevingskans van hoofd-halskankerpatiënten in Nederland de laatste decennia stabiel gebleven op 57%-58%. In diezelfde periode is in Nederland een absolute stijging in incidentie waargenomen van 15% naar 50% voor patiënten met juist een prognostisch gunstig HPV-positief oropharynxcarcinoom, wat nog duidelijker wijst op stagnerende overlevingskansen voor HPV-negatieve gevallen. Dit benadrukt de noodzaak om recidiverende ziekte vroegtijdig op te sporen teneinde de kans op succesvolle curatieve behandeling te verhogen in plaats van het voeren van een palliatief beleid. Met de opkomst van de precisiegeneeskunde is een meer gepersonaliseerde benadering van hoofd-halskanker wenselijk, waarbij nieuwe wegen worden ingeslagen naar meer accurate vroegdetectie van recidiverende ziekte, prognostische stratificatie en behandelresponspredictie. In het ideale geval wordt hierbij gebruikt gemaakt van moleculaire biomarkers die accuraat de (epi)genetische status van de tumor weergeven en tegelijkertijd op minimaal invasieve wijze kunnen worden afgenomen. Tenslotte dient implementatie ervan in de dagelijkse klinische praktijk praktisch haalbaar te zijn. Daarom was het doel van dit proefschrift om potentiële diagnostische biomarkers te identificeren en te onderzoeken of deze geschikt zijn voor klinische implementatie en vroegdetectie van recidiverende hoofd-halskanker, teneinde overlevingskansen en de kwaliteit van leven van hoofd-halskankerpatienten in de toekomst te verbeteren.

Eerdere onderzoeksresultaten van whole-exome sequencing op hoofd-halsplaveisel-celcarcinomen toonde reeds de aanwezigheid van hoge aantallen TP53-mutaties in HPV-negatieve tumoren. Deze bevindingen werden bevestigd in **hoofdstuk 2** door het analyseren van klinisch verkregen next-generation sequencing (NGS) data van meer dan 200 tumoren, waarbij ook subgroepen van primaire tumoren met bijbehorende recidieven en/of metastasen met elkaar werden vergeleken. NGS was oorspronkelijk gebaseerd op 3 verschillende genpanels met hotspots binnen 36-50 genen. TP53-mutaties waren veelvuldig aanwezig in de overgrote meerderheid van HVP-negatieve primaire tumoren en hun bijbehorende recidieven of metastasen die uitsluitend gerelateerd waren aan alcohol en/of roken, terwijl PIK3CA-mutaties vaker werden teruggevonden in niet aan alcohol of roken gerelateerde tumoren. Tevens werd een hoge mate van concordantie van mutaties aangetroffen binnen de genetische profielen van primaire tumoren en hun bijbehorende recidieven of metastasen. Vergeleken met mutaties in andere genen zoals HRAS of PIK3CA, bleken met name TP53-mutaties in klonale uitgroeien vaak synoniem aan de mutaties in de bijbehorende primaire tumor. Minder gevoelige sequencing methoden toonden al eerder vergelijkbare synonymie tussen primaire tumoren en hun klonale uitgroeien. In tegenstelling tot HPV-negatieve primaire tumoren vertonen HPV-positieve recidieven en metastasen meer TP53-mutaties dan de bijbehorende HPV-positieve tumoren. De in hoofdstuk 2 vermelde serie bevatte slechts 4 HPV-positieve tumoren, waarvan geen NGS-data beschikbaar waren van eventuele bijbehorende recidieven of metastasen. Verder bleken CDKN2A- en PIK3CA-mutaties vaak gelijktijdig voor te komen met TP53-mutaties. Deze bevindingen suggereerden om TP53-mutaties in circulerend tumor DNA (ctDNA) te gebruiken als potentiele biomarker, wat een aanmoediging vormde om verder onderzoek te verrichten op het gebied van liquid biopsies in hoofd-halskanker.

In **hoofdstuk 3** werd literatuur over de diagnostische en/of prognostische waarde van celvrije nucleïnezuren in bloed of speeksel systematisch onderzocht ter identificering van studies aangaande behandelmonitoring en prognostiek. De meerderheid van deze studies toonde een statistisch significant verschil in expressiepatroon van één of meerdere microRNA's (miRNA), terwijl slechts 2 studies melding maakten van detecteerbaarheid van ctDNA (RAS-genfamilie) en HPV-DNA in bloed geassocieerd met progressievrije overlevingskans. Een relatief groot aantal studies liet voor verschillende miRNA's een verschil in expressie zien. Deze transcriptiemerkers zijn vaak onderdeel van grote, complexe expressieprofielen bestaande uit meerdere moleculaire targets die genregulatie op indirecte wijze vertegenwoordigen. Dit vereist een veel uitgebreidere analyse vergeleken met 'eenvoudige' detectie en kwantificering van ctDNA. De overige studies beschreven te kleine onderzoeksaantallen of inconclusieve resultaten. Verder bleek het meeste onderzoek op niet-gestandaardiseerde wijze te zijn uitgevoerd wat betreft bloed- en speekselafname en opslag, pre-analytische voorbereiding en analysemethode. Over het algemeen vertoonde dit literatuuronderzoek duidelijk gebrek aan grootschalig longitudinaal onderzoek naar de detectie van ctDNA in bloed of speeksel van hoofd-halskankerpatiënten.

Als reactie op het gebrek aan gepubliceerd onderzoek over ctDNA-analyse bij hoofd-halskankerpatiënten zoals vermeld in hoofdstuk 3, werd in **hoofdstuk 4** onderzocht of

ultragevoelige droplet digital PCR (ddPCR) gebruikt kan worden voor ctDNA-analyse van plasma: DNA werd geïsoleerd uit gearchiveerd, gefixeerd in formaline en ingesloten en in parafine ingesloten (FFPE) primaire tumorbiopten van 6 patiënten met een stadium II-IV HPV-negatieve hoofd-halsplaveiselcelcarcinoom met een TP53-mutatie. Vervolgens werd cfDNA geïsoleerd uit ingevroren plasma dat was afgenomen ten tijde van de diagnose voor opslag in de biobank. Met behulp van ddPCR werd tumor-specifieke analyse van dit plasma verricht op basis van de eerder bepaalde TP53-mutaties. In het plasma van alle patiënten kon ctDNA worden gedetecteerd tot fracties van 0,01% tegen een achtergrond van wildtype cfDNA. Dit bevestigde de technische haalbaarheid van ctDNA-detectie met behulp van ddPCR in plasma van hoofd-halskankerpatiënten.

Om het opwerken van bloed voor ctDNA-analyse verder te verbeteren, werd in **hoofdstuk 5** gezocht naar optimale laboratoriumparameters en -protocollen. Hiertoe werden verschillende methoden en protocollen voor bloedafname, -opslag, -centrifuge en DNA-isolatie en -kwantificering met elkaar vergeleken met behulp van ddPCR-analyse van plasma van gezonde individuen en van longkankerpatiënten. Volbloed dat was opgevangen in siliconen-gecoate stolbuizen voor serumanalyse leverde hogere concentraties aan geïsoleerd DNA op dan in geval bloed werd opgevangen in heparine, citraat en ethyleendiaminetetra-azijnzuur (EDTA) bevattende buizen. Het bloed in stolbuizen was echter vermoedelijk gecontamineerd met grotere hoeveelheden genomisch DNA, aangezien cellen in serum verhoogde kans hebben op lyse ten gevolge van stolling. Verder bleven plasma-cfDNA-concentraties in EDTA-buizen stabiel tot tenminste 24 uur bij opslag op kamertemperatuur. Tweestapscentrifuge verdiende de voorkeur boven enkelvoudige centrifuge om de cellulaire component optimaal te verwijderen. Diverse commercieel beschikbare cfDNA-isolatiekits zoals QIAamp en MagNAPure werkten even goed, maar Zymo Quick leverde de hoogste DNA-concentraties in plasma op. Voor kwantiteitscontrole van DNA-isolaties was Qubit accurater dan Nanodrop.

In lijn van hoofdstuk 4 en 5 werd vervolgens een observationele pilotstudie opgezet, waarvan het doel was om een gestroomlijnde klinische workflow te creëren voor de verzameling en analyse van bloed en speeksel van hoofd-halskankerpatiënten voorafgaand aan en tijdens primaire (chemo)radiatie. In **hoofdstuk 6** werden de voorlopige resultaten getoond van vijf geïncludeerde patiënten van dit lopende onderzoek. Verschillende patronen van veranderingen in ctDNA-concentraties werden tijdens de behandeling bij deze patiënten waargenomen. Deze patronen verschilden tussen de patiënten onderling en tussen bloed en speeksel. Alhoewel geen duidelijke trend herkenbaar was in de analyseresultaten, zijn belangrijke eerste stappen gezet voor de implementatie van praktische onderzoeksprotocollen in de klinische praktijk voor een gestroomlijnde workflow met studiegerelateerde handelingen. Zodoende kan dit ook de aanzet vormen tot de start van nieuwe projecten op het gebied van liquid biopsies. Aanvullende studieresultaten zijn

gewenst die in de toekomst hopelijk verder zullen bijdragen aan nieuwe inzichten inzake het gebruik van liquid biopsies bij hoofd-halskankerpatiënten en die grond kunnen bieden voor verder onderzoek.

Samenvattend heeft dit proefschrift bijgedragen aan het snelgroeiende terrein van liquid biopsies bij hoofd-halskankerpatiënten door een breed scala aan (pre)klinische aspecten te onderzoeken. Dit heeft deuren geopend naar meer doelgericht onderzoek ten aanzien van ctDNA-analyse bij deze patiënten. Alhoewel verschillende aspecten nog onduidelijk zijn, vormt dit proefschrift een leidraad voor verder onderzoek naar het gebruik van liquid biopsies voor gepersonaliseerde kankerzorg in de toekomst, teneinde de levensverwachting en levenskwaliteit van hoofd-halskankerpatiënten op termijn te verbeteren.

A

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CURRICULUM VITAE

Joost Henri van Ginkel was born on the 15th of February, 1985 in The Hague, The Netherlands. After obtaining his gymnasium diploma at the Gymnasium Haganum, he successfully upgraded his graduation marks at Stebo Den Haag in order to bypass numerus fixus for enrollment into medical school at Utrecht University in 2004. During his study, he participated in several extracurricular activities, and followed an internship in pediatrics in Manipal, India. After graduating from medical school at the end of 2011, he started a residency program in radiology. Due to change of interests, Joost quit the program and started his PhD project in 2015 under the supervision of Stefan Willems and Robert van Es at the departments of Pathology and Oral and Maxillofacial Surgery of the University Medical Center Utrecht. In 2016, he was awarded the BOOA Research Grant, and presented several research projects at national and international conferences. In September 2017 he started dental school at Radboud University in Nijmegen, and he will start his residency at the Oral and Maxillofacial Surgery department of the University Medical Center Utrecht in January 2020.

LIST OF PUBLICATIONS

Van Ginkel JH, Huibers MMH, Noorlag R, de Bree R, van Es RJJ, Willems SM. Liquid Biopsy: A Future Tool for Posttreatment Surveillance in Head and Neck Cancer? Pathobiology. 2017

van Ginkel JH, de Leng WW, de Bree R, van Es RJ, Willems SM. Targeted sequencing reveals TP53 as a potential diagnostic biomarker in the post-treatment surveillance of head and neck cancer. Oncotarget. 2016

van Ginkel JH, Huibers MMH, van Es RJJ, de Bree R, Willems SM. Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. BMC Cancer. 2017

van Ginkel JH, Slieker FJB, de Bree R, van Es RJJ, Van Cann EM, Willems SM. 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

van Ginkel JH, van den Broek DA, van Kuik J, Linders D, de Weger R, Willems SM, Huibers MMH. Preanalytical blood sample workup for cell-free DNA analysis using Droplet Digital PCR for future molecular cancer diagnostics. Cancer Med. 2017