Tumor-Informed ctDNA Assay to Predict Outcome in Recurrent and/or Metastatic Squamous Cell Carcinoma of the Head and Neck Treated with PD-1 Inhibitor

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ABSTRACT

Purpose: Although immunotherapy improves the overall survival (OS) of patients with recurrent/metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN), only 15% to 20% of patients derive a long-term benefit. We hypothesized that ctDNA kinetics during immunotherapy, with or without chemotherapy, could predict treatment efficacy in patients with R/M SCCHN.

Experimental Design: Using a personalized, tumor-informed ctDNA assay, we evaluated ctDNA kinetics from pre- to on-treatment (6–10 weeks after treatment initiation) time points. The primary endpoint was the concordance between ctDNA kinetics and best overall response (BOR). Secondary endpoints were the association of ctDNA clearance and kinetics with OS and progression-free survival (PFS).

Results: Of the 41 patients, ctDNA assays were successfully designed for 29, and ctDNA was detected before treatment in 25 patients. ctDNA kinetics was significantly correlated with BOR ($P = 0.00032$). Patients with unfavorable ctDNA kinetics had significantly worse PFS compared with those with favorable ctDNA kinetics (HR = 16.98; 95% confidence interval, 3.35–86.1; $P = 0.0006$). Six patients had ctDNA clearance, all of whom presented a complete or partial response as BOR. PFS and OS were significantly improved in patients with ctDNA clearance compared with those without clearance (PFS: $P = 0.0006$, OS: $P = 0.027$).

Conclusions: ctDNA kinetics, assessed with a tumor-informed assay, can predict anti–PD-1 therapy efficacy in R/M SCCHN. Early ctDNA clearance during the initial weeks of immunotherapy seems to be a strong predictive marker for a favorable response, as well as improved PFS and OS.

Introduction

Immunotherapy has significantly improved patient survival outcomes across various cancer types (1–3). Pembrolizumab, either alone or combined with platinum-based chemotherapy as a first-line treatment, and nivolumab for patients with disease progression following platinum therapy, improve the overall survival (OS) of patients with recurrent/metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN; refs. 4, 5). However, only 15% to 20% of patients will experience long-term clinical benefit from anti–PD-1 therapy (5–7). Although PD-L1 expression on tumor cells and/or immune cells enriches the patient population likely to benefit from anti–PD-1 therapy, reliable biomarkers capable of predicting response to immunotherapy at an early stage are still lacking (8).

ctDNA analysis has shown great promise in better understanding the natural history of cancers in several clinical settings (9, 10). ctDNA is an emerging biomarker to detect minimal residual disease, particularly in virus-induced cancers such as human papillomavirus–positive oropharyngeal carcinomas or Epstein-Barr virus (EBV)-positive nasopharyngeal cancers treated with curative intent (7, 11, 12). In human papillomavirus–negative SCCHN, recent findings suggest that ctDNA can also detect minimal residual disease, using either personalized or non-personalized approaches (12–14). In R/M SCCHN, ctDNA kinetics assessed with a non-personalized assay accurately predicted objective response to PD-1 inhibitors in 74% of patients and was associated with improved progression-free survival (PFS) and OS (15, 16).

Tumor-informed, personalized ctDNA assays, such as the Signatera assay, have already shown robust results in monitoring response to pembrolizumab in several tumor types (17, 18). Bratman and colleagues (19) evaluated the utility of this assay in a cohort of patients receiving pembrolizumab in the INSPIRE phase II study. ctDNA kinetics were evaluated as the difference in ctDNA levels between the initiation of treatment and at the time of the third cure ($\Delta ctDNA_{C3}$) in 73 patients with available plasma. $\Delta ctDNA_{C3}$ was predictive of benefit from pembrolizumab across all cancer types. Out of the 33 patients with favorable $\Delta ctDNA_{C3}$, 14 (42%) achieved an objective response, compared with only 1 (2%) of the 40 patients with unfavorable $\Delta ctDNA_{C3}$. Favorable $\Delta ctDNA_{C3}$ patients showed better OS [HR = 0.36; 95% confidence interval (CI), 0.18–0.71] and PFS (HR = 0.33; 95% CI, 0.19–0.58) across all cancer types. However, only 14 patients with R/M SCCHN were included in Bratman's study (19).

In this study, we evaluated the ability of a tumor-informed, multiplex-PCR, next-generation sequencing (NGS) ctDNA assay to predict anti–PD-1 therapy efficacy in R/M SCCHN. We show that early ctDNA kinetics predict treatment efficacy.

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Translational Relevance

PD-1 inhibitors improve overall survival in patients with recurrent/metastatic squamous cell carcinoma of the head and neck (SCCHN), but only 15% to 20% derive long-term benefit. We investigated whether ctDNA kinetics measured using a personalized, tumor-informed assay could monitor treatment efficacy in patients with SCCHN treated with PD-1 inhibitors ± chemotherapy. ctDNA kinetics significantly correlated with the best overall response ($\chi^2 = 15$, $P = 0.0015$). Patients with unfavorable ctDNA kinetics had significantly worse progression-free survival (HR = 15.11, $P = 0.001$). All six patients with ctDNA clearance achieved a complete or partial response, with significantly improved progression-free survival ($P = 0.007$) and overall survival ($P = 0.033$). Our findings suggest early ctDNA clearance to be a strong predictor of response to PD-1 inhibitors and a valuable predictive biomarker in recurrent/metastatic SCCHN. ctDNA monitoring could be particularly advantageous in a setting in which radiological changes may lag behind biological responses or be confounded by pseudoprogression.

Materials and Methods

Patient cohort, study design, and inclusion criteria

After obtaining written informed consent, all patients with SCCHN treated in our institution with standard-of-care protocols were prospectively enrolled in UCL-ONCO-2013 (2013/12FEV/047), a noninterventional biomarker trial (NCT02139020). The clinical characteristics and disease outcomes of each patient were also prospectively recorded in a secure database [REDCap (20)]. The study was approved by our ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000).

Eligibility criteria for inclusion were as follows: patients (i) enrolled in UCL-ONCO-2013 between 2017 and 2022 with R/M or incurable SCCHN (oral cavity, oropharynx, hypopharynx, larynx, and unknown primary), (ii) treated with noncurative-intent anti–PD-1 therapy with or without concomitant chemotherapy according to the standard of care, (iii) for whom whole blood as well as pre- and on-treatment plasma samples were available, and (iv) treated for at least 6 weeks with anti–PD-1 therapy. The first on-treatment plasma sample to calculate ctDNA kinetics, defined as FU1, had to be collected within 6 to 10 weeks after the initiation of the PD-1 inhibitor. Nasopharyngeal, salivary, and sinonasal cancers were excluded.

Study objective and endpoints

Our main objective was to evaluate if ctDNA kinetics, evaluated by a bespoke multiplex-PCR NGS assay (Signatera, Natera, Inc.), could predict the best overall response (BOR) according to RECIST v1.1. Secondary endpoints were OS and PFS and their association with ctDNA kinetics and clearance.

ctDNA kinetics was defined as the difference in ctDNA levels, measured as mean tumor molecules per milliliter of plasma (MTM/mL), between pretreatment (baseline) and FU1. A threshold of 20% reduction of MTM/mL was used to define the favorable (≥20% MTM/mL reduction) versus unfavorable (<20%

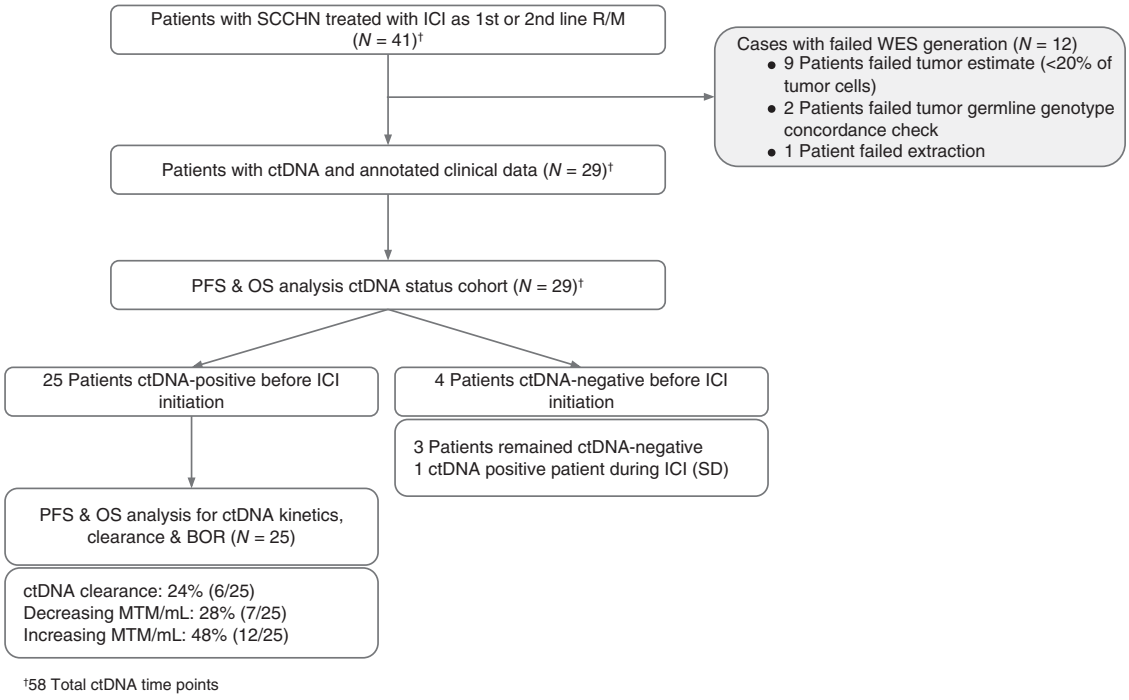


Figure 1.

CONSORT diagram for patient selection and ctDNA analysis. The diagram illustrates patient inclusion and exclusion criteria for the analysis cohort, detailing the reasons for exclusion and summarizing patient groups based on ctDNA positivity prior to and during ICI treatment.

Table 1. Baseline patient and tumor characteristics.

Clinical variable	Favorable ctDNA kinetics *N* = 13	Unfavorable ctDNA kinetics *N* = 12	ctDNA (–) at baseline *N* = 4	Overall *N* = 29 (% of total)
Age				
Median (min, max)	66.5 (41.5, 79.3)	66.2 (46.0, 82.2)	69.2 (44.2, 77.0)	66.6 (41.5, 82.2)
Sex				
Female	4	5	1	10 (34.5%)
Male	9	7	3	19 (65.5%)
Primary tumor location				
Hypopharynx	2	0	1	3 (10.3%)
Larynx	1	0	2	3 (10.3%)
Oral cavity	3	7	0	10 (34.5%)
Oropharynx	7	4	1	12 (41.4%)
p16 status				
Negative	*6*	*2*	*0*	*8 (66.6%)*
Positive	*1*	*2*	*1*	*4 (33.3%)*
Unknown primary	0	1	0	1 (3.4%)
Previous curative-intent treatment regimen				
Primary chemoradiation	4	3	0	4 (30.8%)
Radiotherapy	1	0	0	1 (3.4%)
Primary surgery (± chemoradiation)	2	5	1	8 (27.6%)
No curative intent	6	4	3	13 (44.8%)
Combined positive score				
<1	4	3	1	8 (27.6%)
1–20	3	7	2	12 (41.4%)
≥20	6	2	1	9 (31.0%)
Tobacco use (>10 PY)				
No	3	3	2	8 (27.6%)
Yes	10	9	2	21 (72.4%)
Alcohol use (>2 U/day)				
No	3	5	1	9 (31.0%)
Yes	10	7	3	20 (69.0%)
Eastern Cooperative Oncology Group				
0	9	7	3	19 (65.5%)
1	4	5	0	9 (31.0%)
2	0	0	1	1 (3.4%)
PD-1 inhibitor				
Nivolumab	5	3	1	9 (31.0%)
Pembrolizumab	8	9	3	20 (69.0%)
R/M line in which immunotherapy was administered				
First line	8	7	4	19 (65.5%)
Second line	4	2	0	6 (20.7%)
Third line or more	1	3	0	4 (13.8%)
Disease status before the start of immunotherapy				
Locoregional	8	8	2	18 (62.1%)
Metastatic	5	4	2	11 (37.9%)
Subsequent treatment				
None	10	7	2	19 (65.5%)
Paclitaxel	1	3	2	6 (20.7%)
Unknown	2	1	0	3 (10.3%)
Extreme regimen	0	1	0	1 (3.4%)

reduction or any increase in MTM/mL) ctDNA kinetics groups. BOR was evaluated according to RECIST v1.1 by a radiologist blinded to the ctDNA results. PFS was defined as the time interval between the date of the first day of treatment and the date of SCCHN progression, last follow-up, or death due to any cause. OS was defined as the time interval between the date of the first day of treatment and death due to any cause or until the date of last follow-up.

Blood and plasma collections

Plasma samples were harvested from each patient before the start of anti–PD-1 treatment (baseline sample) and between 6 and 10 weeks after the initiation of treatment (FU1). For each patient and time point, plasma was isolated from whole blood collected in two EDTA 7.5 mL tubes after double centrifugation (2,000 *g* for 10 minutes and 2,500 *g* for 15 minutes) within 30 minutes at 4°C. Plasma was stored immediately at −80°C until further use. Two to

eight milliliters of plasma was used for DNA extraction, performed as previously described (15).

PD-L1 IHC and combined positive score

Formalin-fixed, paraffin-embedded tumor specimens were stained for PD-L1 using the FDA-approved 22C3 pharmDx assay (Dako, Inc.) on the BenchMark VENTANA automated platform with the OptiView DAB IHC Detection Kit, following the manufacturer's protocol, as previously described (21). PD-L1 expression was quantified by the combined positive score, defined as PD-L1–staining cells (including tumor cells, lymphocytes, and macrophages), divided by the total number of viable tumor cells in the same specimen, and then multiplied by 100 to yield a percentage. A combined positive score ≥1 was considered PD-L1–positive in accordance with published guidelines.

Germline DNA extraction

Matched germline DNA was extracted for each patient with the Promega Wizard Genomic DNA Purification kit [Rapid Sample Concentrator (RSC); Promega] from a whole blood sample in EDTA 3.5 mL tubes, frozen at −80°C, and quantified with NanoDrop technology (Thermo Fisher Scientific). At least 40 μL of germline DNA was used for extraction, as previously described (15).

Tumor preparation

For each patient, 5 to 10 unbaked slides at 7 μm from needle core biopsy were used for tumor DNA (tDNA) extraction, accompanied by one fixed slide colored with hematoxylin and eosin for tumor-contouring purposes. Only samples containing more than 20% of tumor cells were deemed to have sufficient tDNA to be extracted. tDNA was extracted as previously described (19).

Personalized ctDNA assays

Personalized and tumor-informed ctDNA assays (Signatera RUO, Natera, Inc.) were designed and used as previously described (22, 23). Briefly, whole-exome sequencing (WES) was performed on formalin-fixed, paraffin-embedded tumor tissue biopsies and matched normal blood samples. Based on WES results, up to 16 patient-specific somatic SNVs were selected for each patient for downstream plasma-based ctDNA testing. Plasma samples with ≥2 SNVs above a predefined confidence threshold were defined as ctDNA-positive, and ctDNA concentration was reported in MTM/mL of plasma.

Analysis of genomic alterations

Somatic variants from Natera's proprietary calling pipeline were annotated using VEP (RRID:SCR_007931; v105). The mutation annotation format files were filtered to exclude known frequently mutated genes and retain nonsynonymous variants with moderate or high impact on protein function (24). The top 25 most commonly mutated genes and proteins were used to generate oncoplots to display mutation frequencies. Analyses were performed using R (RRID:SCR_001905; v4.5.0) and maftools (RRID:SCR_024519; 2.24).

Statistics

Concordance between ctDNA kinetics and BOR in patients was evaluated using Fisher's exact test. We hypothesized that

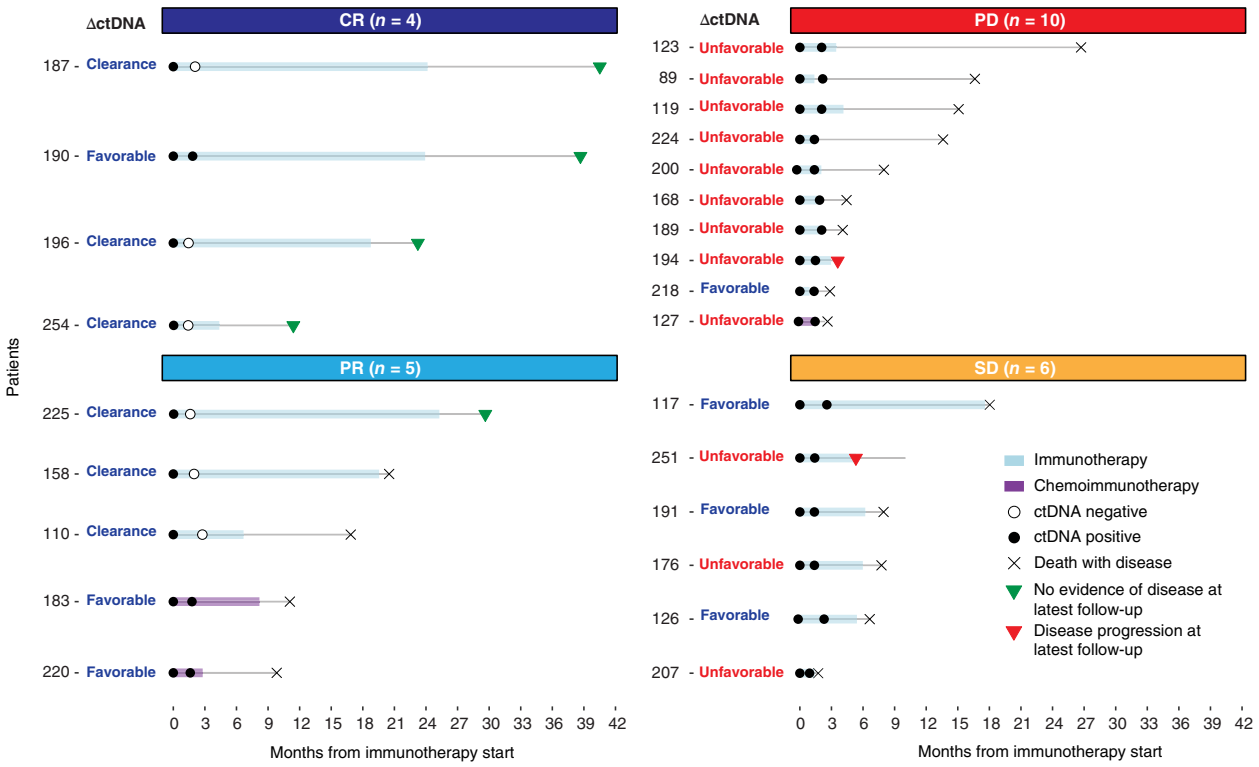
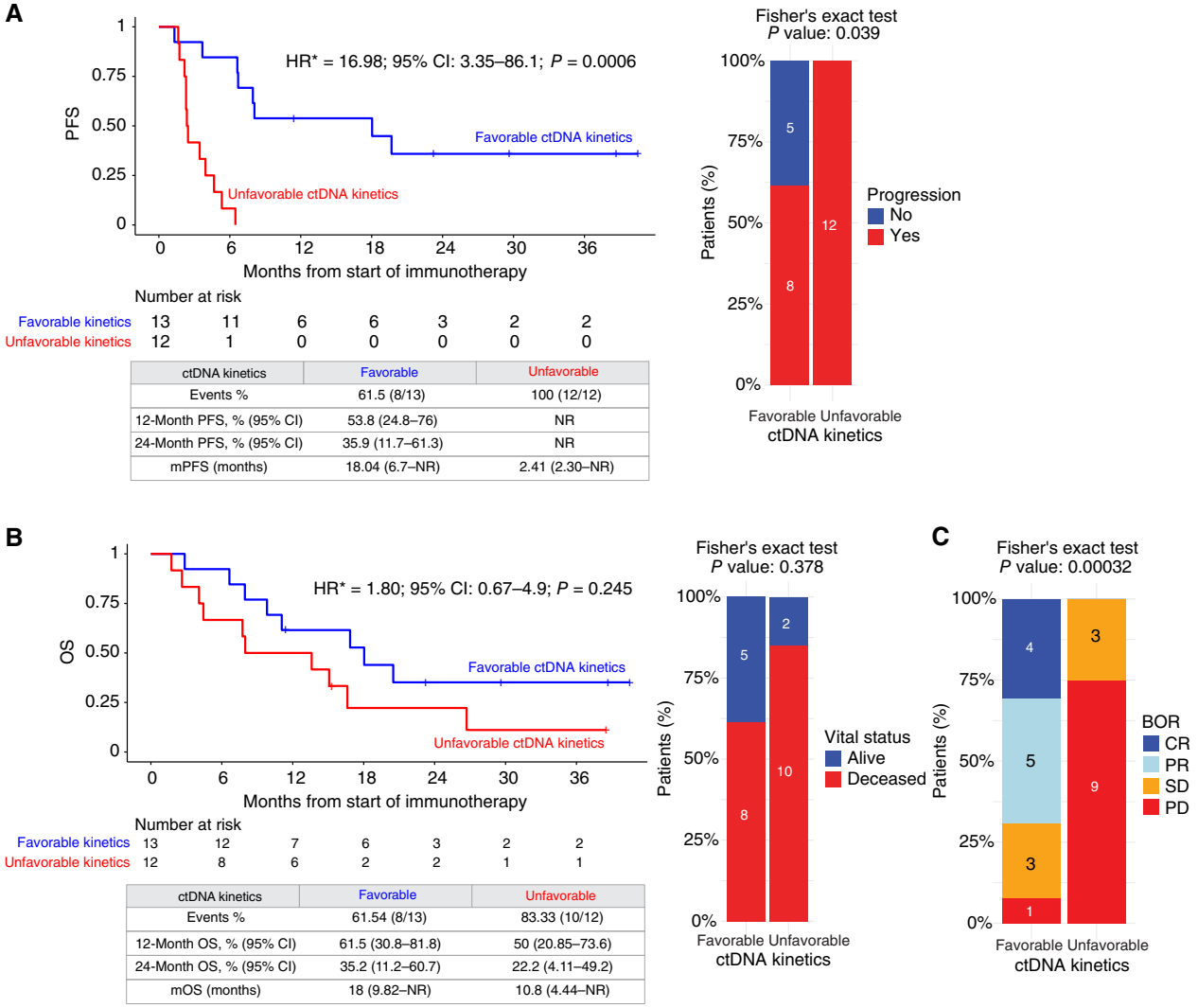


Figure 2.

Swimmer plot stratified by BOR and ctDNA kinetics. Patient 89 received chemotherapy (paclitaxel) following the completion of immunotherapy.



ctDNA kinetics	Favorable	Unfavorable
Events %	61.5 (8/13)	100 (12/12)
12-Month PFS, % (95% CI)	53.8 (24.8–76)	NR
24-Month PFS, % (95% CI)	35.9 (11.7–61.3)	NR
mPFS (months)	18.04 (6.7–NR)	2.41 (2.30–NR)

ctDNA kinetics	Favorable	Unfavorable
Events %	61.54 (8/13)	83.33 (10/12)
12-Month OS, % (95% CI)	61.5 (30.8–81.8)	50 (20.85–73.6)
24-Month OS, % (95% CI)	35.2 (11.2–60.7)	22.2 (4.11–49.2)
mOS (months)	18 (9.82–NR)	10.8 (4.44–NR)

Figure 3.
Kaplan–Meier estimates for PFS (**A**) and OS (**B**) stratified by ctDNA kinetics. (*Continued on the following page.*)

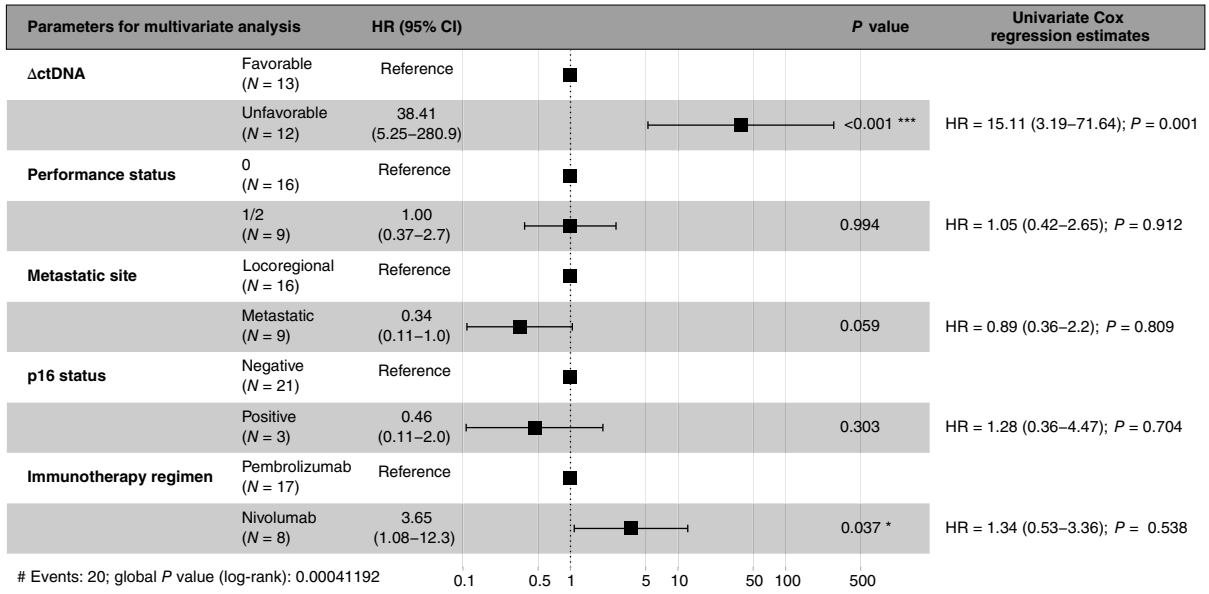
patients with favorable ctDNA kinetics would achieve either a complete response (CR) or partial response (PR) as BOR, whereas patients with unfavorable ctDNA kinetics would experience stable disease (SD) or progressive disease (PD) as BOR. We compared the proportion of patients who fulfilled these criteria with the patients who did not fulfill them. Patient characteristics were summarized using descriptive statistics. Survival probability curves were estimated using the Kaplan–Meier method; HRs were adjusted for Eastern Cooperative Oncology Group performance status, p16 status, and the location of recurrence (locoregional only vs. distant metastases). The log-rank test was used to compare two survival distributions. Overall, *P* values ≤0.05 were considered statistically significant. A multivariable Cox proportional hazards model assessed prognostic factors associated with PFS and OS. All analyses were done using R [RRID: SCR_001905; v4.5.0; R packages survminer (RRID:SCR_021094) v0.4.9 and survival (RRID:SCR_021137) v3.2.13; ref. 25].

Results

Patient characteristics

Between January 2019 and December 2021, 50 patients with R/M SCCHN treated with immune checkpoint inhibitors (ICI) were enrolled in the UCL-ONCO-2013 biomarker trial. Forty-one patients met our inclusion criteria (**Fig. 1**). Among the 41 patients, the assay was successfully designed for 29 patients. Twelve patients failed tumor WES generation: nine due to an insufficient tumor cell proportion in the biopsy sample (<20%), one due to failed DNA extraction, and two due to a failed tumor germline genotype concordance check. The clinical characteristics of these 29 patients are summarized in **Table 1** and compared with the broader SCCHN population in Supplementary Table S1. The most common primary location was the oropharynx [12/29 (41.4%)]. The majority of patients received pembrolizumab [20/29 (69%)], including three patients who received it in combination with chemotherapy. The median OS for the 29 patients was 14.4 (95% CI, 9.82–26.7) months, and the median PFS was 5.32 (95% CI, 3.45–8.08) months.

D



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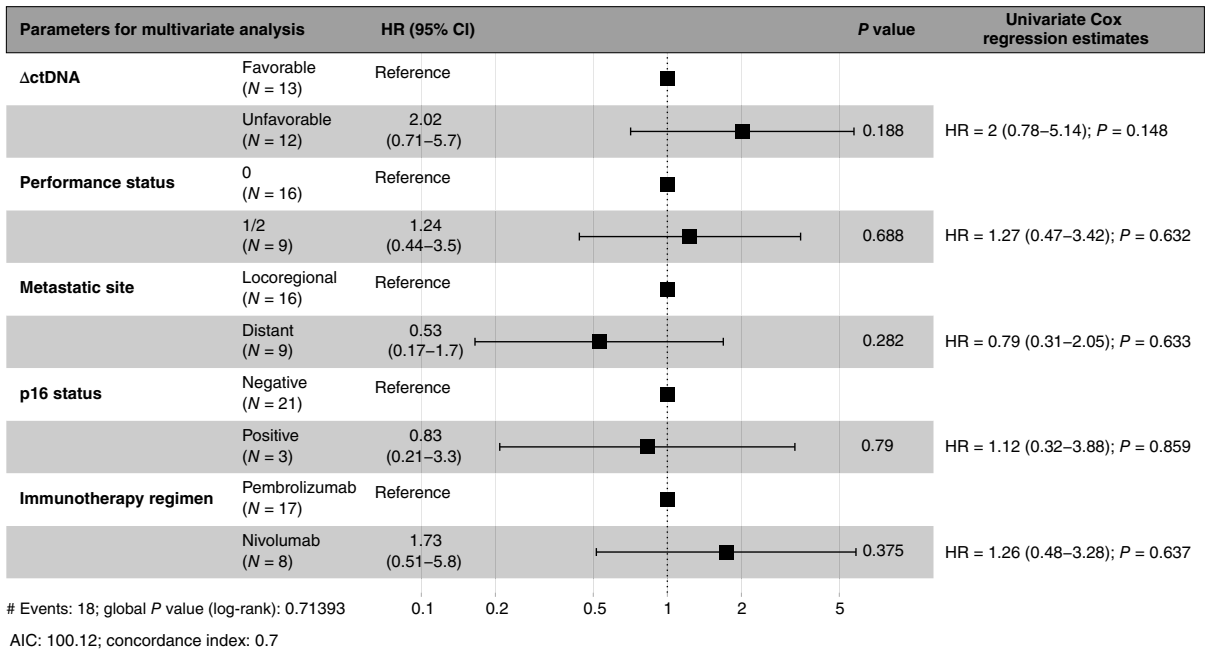
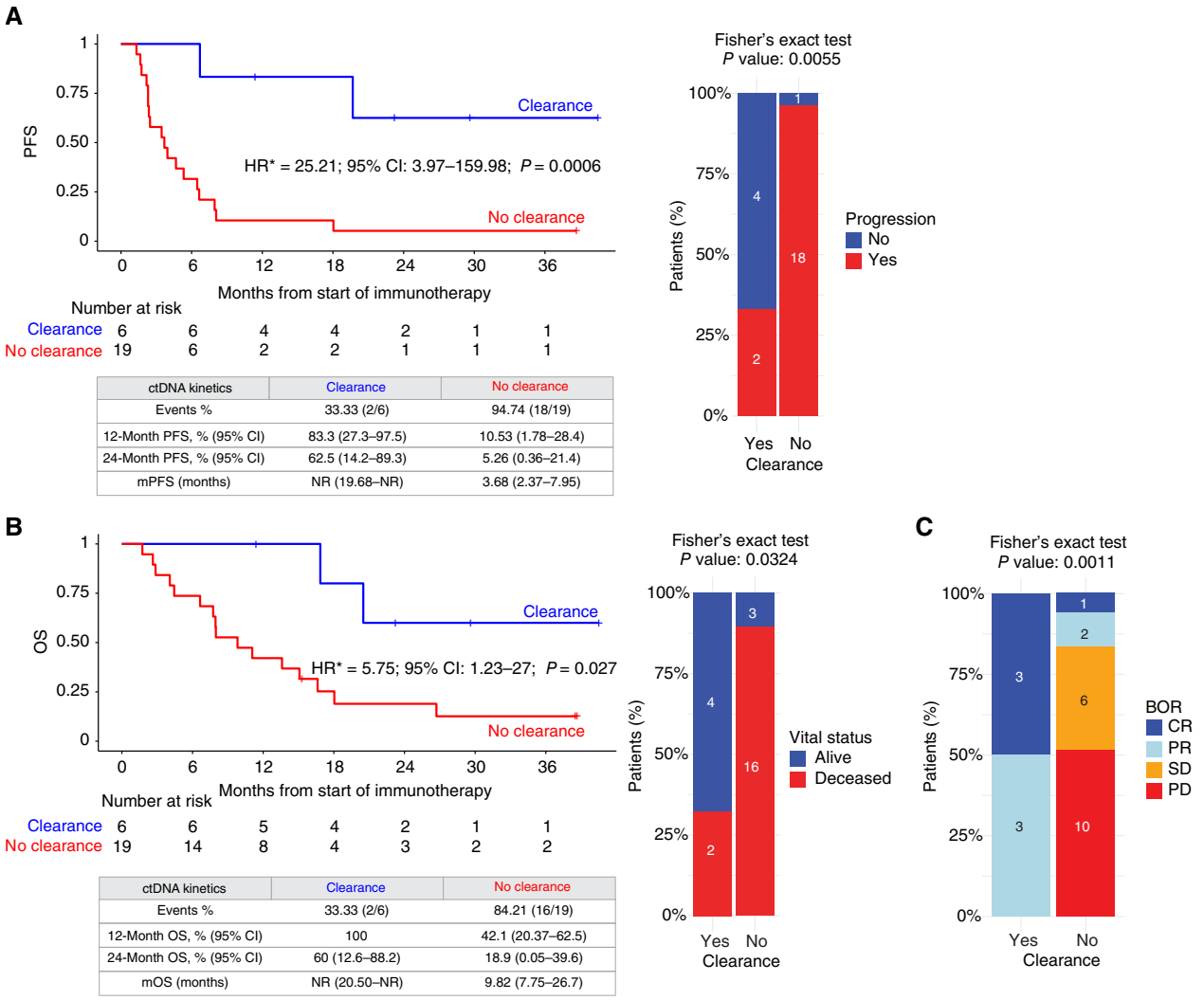


Figure 3.
(*Continued.*) **C,** BOR (CR, PR, SD, and PD) stratified based on ctDNA kinetics. Forest plot depicting the multivariate analysis (including ctDNA kinetics and other clinicopathologic factors) for PFS (**D**) and OS (**E**). In **A** and **B**, HRs for PFS and OS have been adjusted for Eastern Cooperative Oncology Group (0 vs. 1/2), p16 status (negative vs. positive), and the location of recurrence (locoregional only vs. distant metastases). Favorable ctDNA kinetics was defined as a >20% decrease in ctDNA levels; unfavorable ctDNA kinetics included a <20% decrease or any increase in ctDNA levels. NR, not reached. AIC, Akaike information criterion.

Tumor-personalized assay detects ctDNA

Out of the 29 patients, ctDNA was not detected in four patients in the pretreatment samples. These four patients had "quality control" flags related to extracted cfDNA concentration/assay design, which may have contributed to these negative results. Therefore, in the pretreatment setting, ctDNA was detected in 25 (86.2%) out of the 29 plasma samples. The median ctDNA level was 8.4 MTM/mL (range: 0.2–986). Of note, among the four patients with



ctDNA kinetics	Clearance	No clearance
Events %	33.33 (2/6)	94.74 (18/19)
12-Month PFS, % (95% CI)	83.3 (27.3–97.5)	10.53 (1.78–28.4)
24-Month PFS, % (95% CI)	62.5 (14.2–89.3)	5.26 (0.36–21.4)
mPFS (months)	NR (19.68–NR)	3.68 (2.37–7.95)

ctDNA kinetics	Clearance	No clearance
Events %	33.33 (2/6)	84.21 (16/19)
12-Month OS, % (95% CI)	100	42.1 (20.37–62.5)
24-Month OS, % (95% CI)	60 (12.6–88.2)	18.9 (0.05–39.6)
mOS (months)	NR (20.50–NR)	9.82 (7.75–26.7)

Figure 4.

Kaplan–Meier estimates for PFS (**A**) and OS (**B**) stratified by ctDNA clearance upon ICI treatment. **C,** BOR (CR, PR, SD, and PD) stratified based on ctDNA kinetics.
(*Continued on the following page.*)

undetectable ctDNA, one patient had ctDNA detected during treatment (plasma sample procured 10 weeks after ICI initiation). This patient had SD as BOR. The other three patients remained ctDNA negative, two with PD as BOR and one with CR. During FU1, ctDNA was detected in 19 out of 25 (76.0%) plasma samples, with a median ctDNA level of 7.9 MTM/mL (range: 0.1–737.0).

ctDNA kinetics is associated with BOR and predicts long-term response to ICI

The primary endpoint was to evaluate whether ctDNA kinetics could predict BOR according to RECIST v1.1. Only the 25 patients with ctDNA detected at baseline were included in this analysis. Among these patients, the objective response rate was 36.0% (9/25), with four patients (16.0%) achieving CR and five patients (20.0%) achieving PR. Six patients (24.0%) achieved SD, whereas 10 (40%) had PD (**Fig. 2**).

ctDNA kinetics were statistically correlated with BOR ($P = 0.00032$) and predicted BOR in 84.0% (21/25) of patients treated with anti–PD-1 therapy with or without chemotherapy. Thirteen (52.0%) patients had a ≥20% decrease in ctDNA levels at the FU1 time point, collected between weeks 6 and 10 after anti–PD-1 therapy initiation (favorable ctDNA kinetics group), among which 6 (24.0% of the total cohort) had complete ctDNA clearance. In the favorable ctDNA kinetics group, nine patients achieved an objective response (four CR and five PR), three had SD, and one experienced PD. Twelve patients (48.0%) were in the unfavorable ctDNA kinetics group (any increase or <20% decrease in ctDNA levels), none of whom had an objective response to PD-1 inhibitors (**Fig. 2**). Notably, all patients with increasing ctDNA kinetics experienced SD (3/12, 25%) or PD (9/12, 75%), independent of the immunotherapy regimen (nivolumab vs. pembrolizumab; Supplementary Fig. S1A and S1B). Three patients received chemotherapy in addition to pembrolizumab. Two of them were in the favorable ctDNA kinetics group and had a PR, whereas the third patient, in the unfavorable ctDNA kinetics group, experienced PD.

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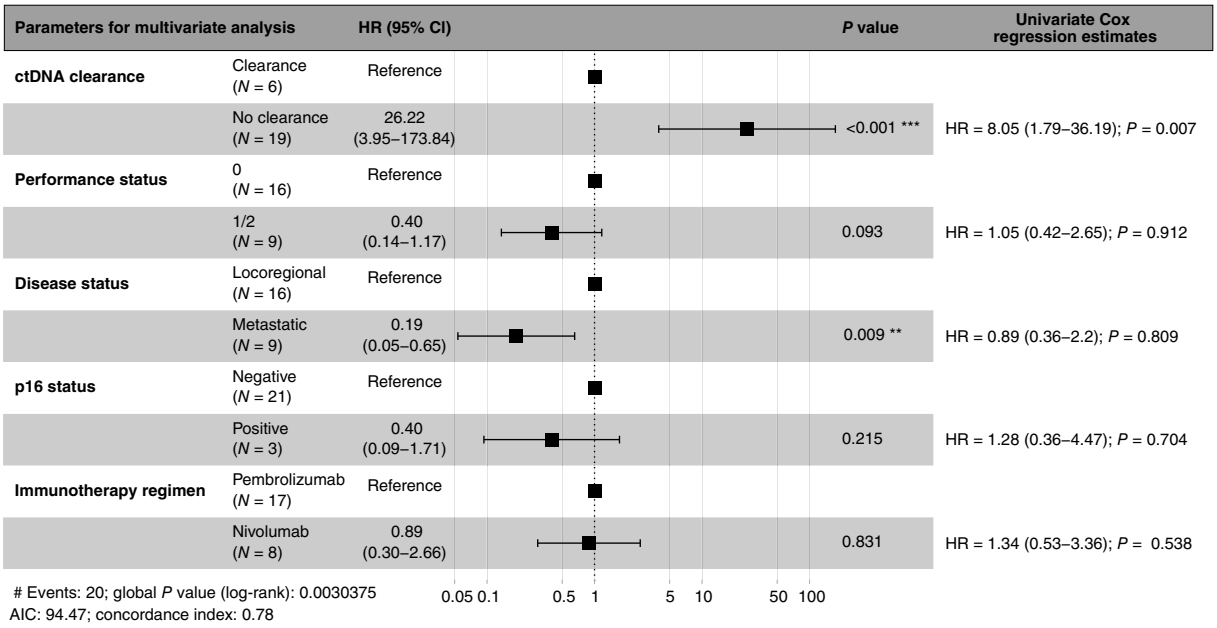

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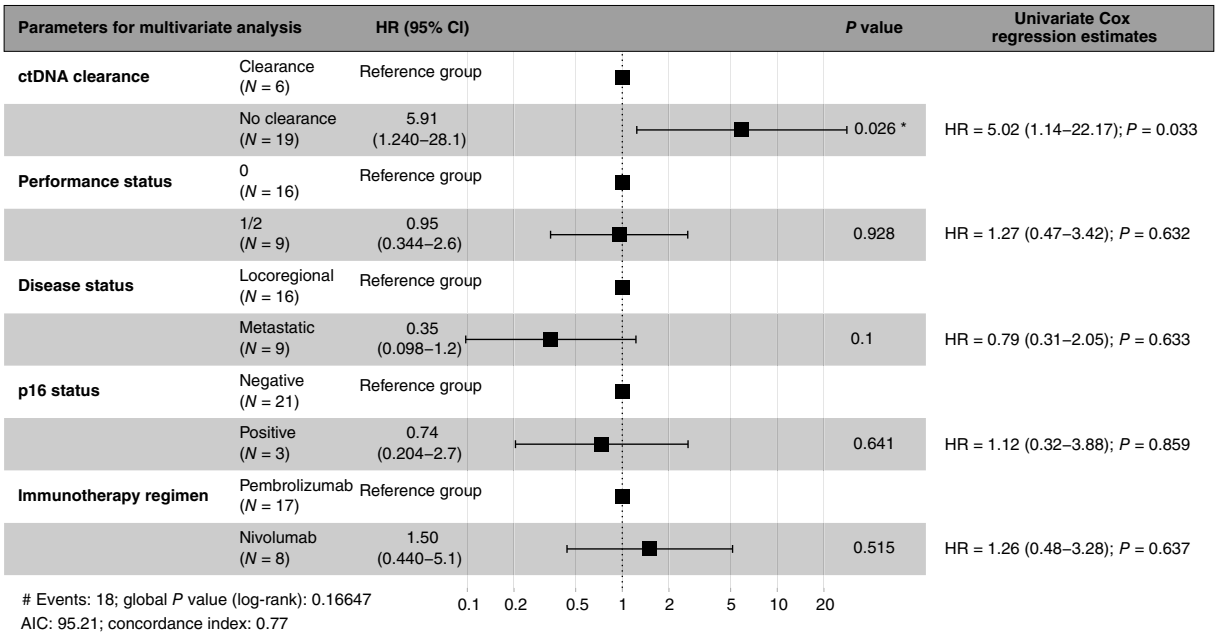

Figure 4.
(*Continued.*) Forest plot depicting the multivariate analysis (including ctDNA clearance and other clinicopathologic factors) for PFS (**D**) and OS (**E**). In **A** and **B**, HRs for PFS and OS have been adjusted for Eastern Cooperative Oncology Group (ECOG; 0 vs. 1/2), p16 status (negative vs. positive), and the location of recurrence (locoregional only vs. distant metastases). NR, not reached, AIC, Akaike information criterion.

When evaluating ctDNA kinetics, patients who had unfavorable ctDNA kinetics had significantly worse PFS when compared with those with favorable ctDNA kinetics (PFS: HR = 16.98; 95% CI, 3.35–86.10; $P = 0.0006$; **Fig. 3A**). However, there was no statistical difference in the OS (HR = 1.80; 95% CI, 0.67–4.9; $P = 0.245$) between these groups (**Fig. 3B**). Consistently, all patients who showed an increase in ctDNA levels had PD or SD ($P = 0.00032$; **Fig. 3C**). The multivariate analyses demonstrated ctDNA kinetics to be the strongest prognostic factor for PFS (HR = 38.41; 95% CI, 5.25–280.90; $P < 0.001$), with the immunotherapy regimen (nivolumab) being the only other clinicopathologic factor significantly prognostic of PFS (HR = 3.65; 95% CI, 1.08–12.3; $P = 0.037$;

Fig. 3D). None of the factors were prognostic of OS in the multivariate analysis (**Fig. 3E**).

ctDNA status and clearance on posttreatment samples predict long-term outcomes to ICI

Among 29 patients with personalized ctDNA assays designed, we investigated whether the absence of ctDNA detected at FU1 (ctDNA status) was associated with better PFS, OS, and objective response rate. At FU1, ctDNA was undetectable in nine patients, whereas 20 patients were ctDNA-positive at this time point. The median PFS and OS were not reached in ctDNA-negative patients at FU1 and were 3.81 and 10.4 months, respectively, in ctDNA-positive patients. Furthermore, ctDNA-positive patients at FU1 had a significantly shorter PFS and OS when compared with ctDNA-negative patients (PFS: HR = 5.79; 95% CI, 1.59–21; $P = 0.008$; OS: HR = 3.25; 95% CI, 1.01–10.5; $P = 0.048$; Supplementary Fig. S2A and S2B). Notably, 50% (10/20) of the ctDNA-positive patients at FU1 had PD, and 35% (7/20) had SD. In comparison, only 22% (2/9) of ctDNA-negative patients at FU1 had PD, whereas none had SD ($P = 0.0065$; Supplementary Fig. 2C).

Of the 25 patients who were ctDNA-positive at baseline, we investigated whether ctDNA clearance was associated with clinical outcome. Six had complete ctDNA clearance. These patients had a significantly improved PFS (HR = 25.21; 95% CI, 3.97–159.98; $P = 0.0006$; **Fig. 4A**) and OS (HR = 5.75; 95% CI, 1.23–27; $P = 0.027$; **Fig. 4B**) compared with the 19 patients who did not achieve ctDNA clearance. Median PFS and OS were not reached in the clearance group and were 3.68 and 9.82 months, respectively, in the no-clearance group. Moreover, 94.7% (18/19) of patients without ctDNA clearance eventually showed PD, and 84.2% (16/19) succumbed to the disease. Evaluating BOR based on ctDNA clearance, we observed that the majority of patients (84.2%, 16/19) who failed to clear their ctDNA had PD or SD; in contrast, all patients who achieved ctDNA clearance (100%, 6/6) had CR or PR (**Fig. 4C**; $P = 0.0011$). Multivariate analysis revealed that ctDNA clearance during treatment was the only factor significantly associated with better PFS ($P < 0.001$) and OS ($P = 0.026$) in a model including Eastern Cooperative Oncology Group score, clinical stage (patients with metastases vs. no metastases), p16 status, and immunotherapy regimen (**Fig. 4D** and **E**).

Genomic alterations associated with clinicopathologic features and ctDNA clearance

To further elucidate any specific genomic alterations associated with differential clinical outcomes or distinct ctDNA kinetic patterns following anti–PD-1 therapy, we performed an integrated analysis comparing baseline tissue mutational profiles, ctDNA dynamics, and clinical endpoints. WES of tumor tissue revealed that the most frequently mutated genes were *TP53* (76%), *CDKN2A* (31%), and *FAT1* (21%; Supplementary Fig. S3).

Upon reviewing clinical and genomic annotations per patient, common mutations such as *TP53*, *CDKN2A*, *FAT1*, and *NOTCH1* did not demonstrate a clear correlation with ctDNA kinetics or clinical outcomes (**Fig. 5**). This integrated visualization underscores the robust predictive capability of early ctDNA kinetics for clinical outcome stratification in patients treated with ICIs.

Discussion

This prospective biomarker study demonstrates that early changes in ctDNA levels, measured via a tumor-informed assay, are significantly predictive of response to anti–PD-1 therapy in patients with R/M SCCHN.

ctDNA kinetics from pretreatment to the 6- to 10-week treatment time point predicted BOR in 84.0% (21/25) of patients treated with anti–PD-1 with or without chemotherapy. Furthermore, patients with favorable ctDNA kinetics had significantly better PFS compared with those with unfavorable ctDNA kinetics, which is consistent with previous studies in SCCHN (16, 19). Although no significant OS difference was observed between the unfavorable and favorable ctDNA kinetics groups, ctDNA kinetics was the strongest prognostic factor of PFS in the multivariate analysis. Importantly, ctDNA clearance emerged as an even more robust biomarker of therapeutic efficacy. Despite being observed in only 24% (6/25) of patients, all six patients with complete ctDNA clearance by the first follow-up time point within 6 to 10 weeks of anti–PD-1 therapy exhibited an objective response, whereas only 15.7% (3/19) of those without ctDNA clearance did. Moreover, ctDNA clearance was significantly associated with prolonged PFS and OS, emerging as the only statistically significant predictor for both endpoints in the multivariable analyses. To the best of our knowledge, this is the first evidence of improved survival in SCCHN associated with ctDNA clearance.

Tumor-informed ctDNA monitoring has the potential to assist clinicians in anticipating responses to PD-1 inhibitors and adjusting treatment strategies accordingly. Patients with unfavorable ctDNA kinetics had a shorter median PFS (2.4 months) than those with favorable kinetics (18.0 months). In our study, ctDNA kinetics was able to identify the patients who experienced primary resistance to immunotherapy within the first 6 to 10 weeks of treatment. Previous studies have shown ctDNA dynamics measured as early as within 1 to 4 weeks of treatment initiation to be predictive of anti–PD-1 therapy benefit (16, 19, 26). However, there are no data indicating the best sampling time to evaluate ctDNA kinetics or clearance in SCCHN. In this study, we anchored our ctDNA testing to the standard imaging window to ensure that the ctDNA kinetics could be directly correlated with clinical assessments and PFS. This ctDNA testing window reflects a pragmatic and clinically translatable strategy that is also consistent with other published immunotherapy studies (27–30). Further studies evaluating ctDNA kinetics and clearance at earlier time points are warranted, which could facilitate earlier treatment changes or escalation among nonresponders with the aim of increasing OS for these patients.

Although PD-L1 expression has been adopted as a predictive biomarker in SCCHN, its limitations are well documented, including spatial and temporal heterogeneity, assay variability, and modest predictive performance (31). Additionally, dynamic blood-based biomarkers may offer greater clinical relevance and superior predictive performance than static tissue-based assays. In our study, ctDNA kinetics and clearance were found to be strongly predictive of response to anti–PD-1 therapy in patients with R/M SCCHN. ctDNA monitoring could also be advantageous in a setting in which radiological changes may lag behind biological responses or be confounded by pseudoprogression (29, 32, 33). Although pseudoprogression is a rare phenomenon in R/M SCCHN treated with immunotherapy, more data are needed to assess the potential of ctDNA in that setting.

Of 41 patients, only 29 (70.7%) could be evaluated with personalized ctDNA assays. Ten of the patients were not eligible for this methodology, either because of low tumor cell proportion in the biopsy for assay design or failed tDNA extraction. Out of those 10 patients, 60% (6/10) had primary tumor archival biopsy, rather than recurrent/metastatic tumor, as a tissue source. Despite the

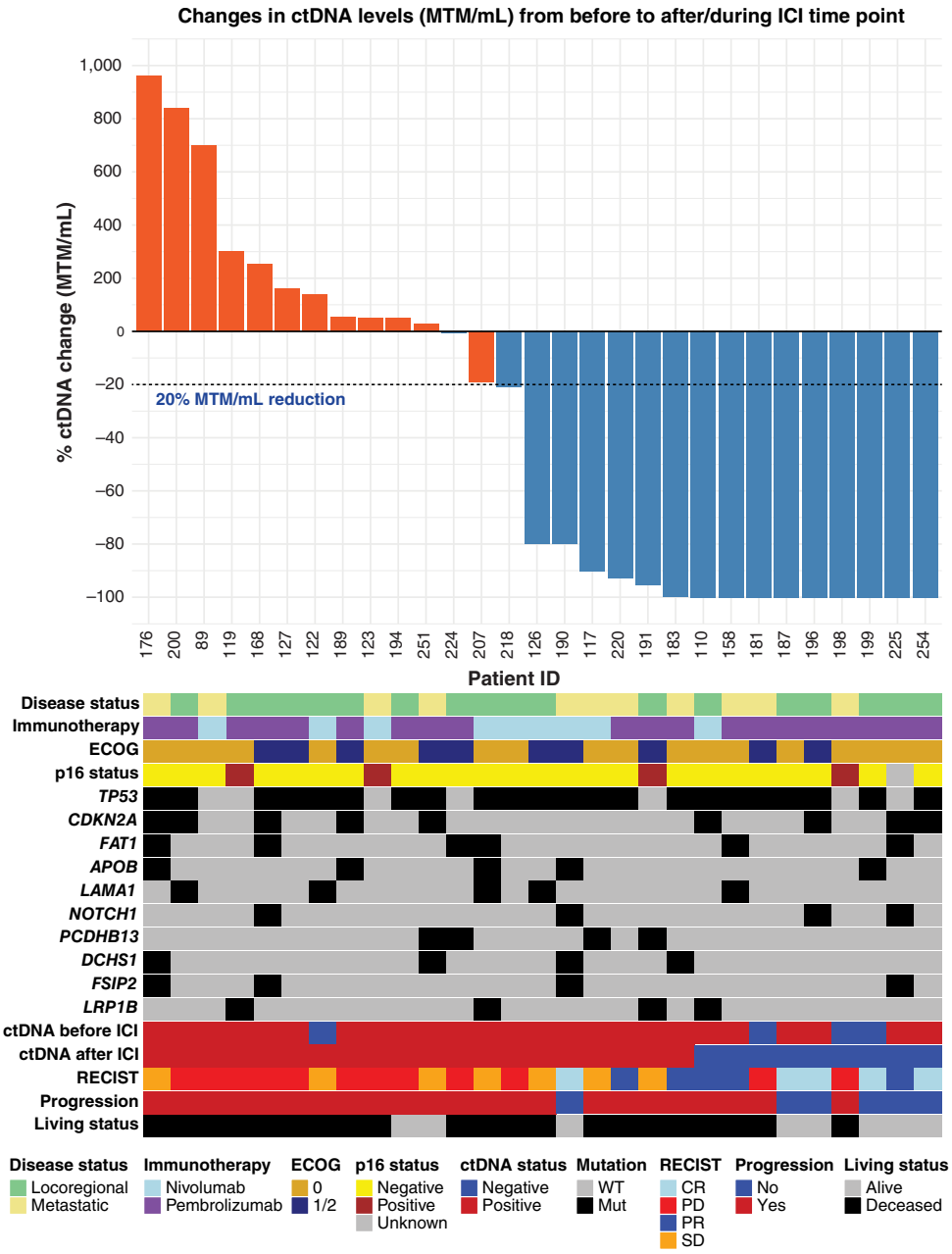

Figure 5.

ctDNA dynamics and clinical outcomes following ICI therapy. Waterfall plot depicting the percentage change in ctDNA levels from baseline (before treatment) to the first on-treatment measurement (during/after ICI). Patients were categorized based on achieving a threshold of ≥20% reduction in ctDNA levels, indicated by the horizontal dashed line. Bars are color-coded to reflect clinical outcomes: Blue bars indicate patients with ≥20% ctDNA reduction, and orange bars indicate patients with <20% decrease in or increasing ctDNA levels. The heatmap below provides patient-specific annotations, including disease extent (locoregional vs. metastatic), immunotherapy type (nivolumab vs. pembrolizumab), Eastern Cooperative Oncology Group performance status, p16 status, specific gene mutation profiles, ctDNA positivity before and after ICI, RECIST response categories (CR, PR, SD, PD), radiologic progression status, and survival status.

strong concordance observed between ctDNA kinetics and clinical outcomes among evaluable patients, it is important to note that ctDNA was not detected before treatment among 13.8% (4/29) of patients. Interestingly, samples from all four of these patients had quality control flags related to extracted cell-free DNA concentration and/or assay design, which may have contributed to the negative result. Another potential reason for the lack of baseline ctDNA positivity is lower ctDNA shedding in SCCHN compared with some solid tumors, such as colorectal cancer (34), as well as the variability in ctDNA levels within SCCHN arising from factors such as the location of the primary tumor site (13, 35). This biological nuance reinforces the importance of using highly sensitive tumor-informed assays for SCCHN.

Although our study was prospectively designed, several limitations merit discussion. First, the modest cohort size, particularly for those with ctDNA clearance ($n = 6$), restricts definitive conclusions about prognostic implications. Second, the treatment regimen in the cohort was heterogeneous in terms of both anti–PD-1 agent (nivolumab vs. pembrolizumab) and chemotherapy use. Nonetheless, multivariate analyses adjusted for these factors, and the predictive role of ctDNA dynamics remained significant. Due to the study's noninterventional design, the impact of early nonresponse prediction on treatment changes and OS improvement was not evaluated. Finally, although our study focused on a single time point for on-treatment assessment, longitudinal monitoring may further enhance predictive

power and enable the detection of progression or the development of treatment resistance.

In conclusion, our findings support the clinical utility of longitudinal ctDNA monitoring as a dynamic and minimally invasive tool to assess treatment efficacy and predict survival outcomes among patients with R/M SCCHN treated with anti–PD-1 inhibitors with or without chemotherapy, complementing conventional imaging modalities. In the era of personalized medicine, ctDNA monitoring will play an important role in identifying patients who need early treatment adaptation or modification.

Data Availability

All relevant data used to conduct the analyses are available within the article. To protect the privacy and confidentiality of patients in this study, identifiable clinical data are not made publicly available in a repository or in the supplementary material of the article, but can be requested from the corresponding author. Any requests will be reviewed within a time frame of 2 to 3 weeks by the corresponding author to verify whether the request is subject to any intellectual property or confidentiality obligations. All data shared will be deidentified. The fully documented code for the R statistical computing environment for analyses related to this article is deposited in the GitHub repository and can be accessed at https://github.com/Natera-TMED/Honore-et-al_HNSCC-EORTC-IO.git.

Authors' Disclosures

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Authors' Contributions

N. Honoré: Conceptualization, resources, data curation, software, formal analysis, investigation, methodology, writing–original draft. **G. Laliotis:** Software, formal analysis, visualization, writing–review and editing. **V.N. Aushev:** Software, writing–review and editing. **S. Velichko:** Methodology, writing–review and editing. **C.C. Palsuledesai:** Visualization, project administration, writing–review and editing. **H. Dahou:** Data curation. **C. van Marcke:** Conceptualization, writing–review and editing. **R. Galot:** Conceptualization, writing–review and editing. **M.C. Liu:** Supervision, project administration, writing–review and editing. **J.-P. Machiels:** Conceptualization, formal analysis, supervision, validation, investigation, visualization, methodology, writing–original draft, project administration.

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Note

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