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Original Research

Tumour-agnostic plasma assay for circulating tumour DNA predicts outcome in recurrent and/or metastatic squamous cell carcinoma of the head and neck treated with a PD-1 inhibitor

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ABSTRACT

Background: Only 15–20% of recurrent and/or metastatic squamous cell carcinoma of the head and neck (R/M SCCHN) patients derive long-term benefit from nivolumab or pembrolizumab. We developed a circulating tumour DNA (ctDNA) tumour-agnostic assay aimed at the early prediction of single agent programmed cell death 1 (PD1) inhibitor efficacy in R/M SCCHN.

Patients and methods: Our tumour-agnostic assay included 37 genes frequently mutated in R/M SCCHN and two HPV16 genes. Primary endpoint was the concordance between ctDNA kinetics (ΔctDNA) and the best overall response according to Response Evaluation Criteria in Solid Tumors version 1.1. ΔctDNA was defined as the difference in mean variant allele frequency (VAF) between the on-treatment sample harvested 6–10 weeks (FU1) after PD1 inhibitor initiation and the pre-treatment plasma sample (ΔctDNA = mean FU1 VAF - mean pre-treatment VAF).

Results: ctDNA was detected in 35/44 (80%) of the pre-treatment plasma samples. The concordance between ΔctDNA and imaging response was observed in 74%. Median progression-free survival was 8.6 months in the favourable ΔctDNA group and 2.5 months in the unfavourable ΔctDNA group ($p = 0.057$). Median overall survival (OS) was 18.1 and 8.2 months in the favourable and unfavourable ΔctDNA groups, respectively ($p = 0.13$). In patients with PD-L1 expressing SCCHN (Combined Positive Score ≥ 1), OS was significantly better in patients with favourable ΔctDNA compared with patients with unfavourable ΔctDNA: median OS was 41.5 and 8.4 months ($p = 0.033$), respectively.

Conclusions: Tumour-agnostic ctDNA analysis for human papillomavirus (HPV)-negative and HPV-positive R/M SCCHN is feasible. ctDNA kinetics show promising results in predicting the efficacy of PD1 inhibitors in R/M SCCHN.

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1. Introduction

Although PD1 inhibitors are now standard of care in recurrent and/or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN), only around 15–20% of patients will derive long-term benefit from this therapy. [1] PDL-1 expression is the only marker currently used in daily clinical practice that selects patients who have a higher probability of response to a PD1 inhibitor in R/M SCCHN. [2,3].

Circulating tumour DNA (ctDNA) kinetics are being investigated as a biomarker to predict immunotherapy efficacy [4–8] with the hypothesis that changes in ctDNA quantity could early identify patients likely to progress rapidly or derive long-term benefit. Bratman et al. [9] showed that early ctDNA kinetics could predict pembrolizumab benefit in several tumour types using a tumour-informed assay, but only 14 R/M SCCHN patients were included in the study. In a small cohort of R/M human papillomavirus (HPV)-driven oropharyngeal cancer (n = 12), Haring et al. [10] found that longitudinal changes in HPV16 ctDNA measured by droplet digital polymerase chain reaction correlated with response to chemotherapy and/or immunotherapy. Using a tumour-agnostic assay (CAncer Personalized Profiling by deep Sequencing (CAPP-Seq)), Taylor et al. [11] observed that early dynamic changes in ctDNA level predicted both overall survival (OS) and progression-free survival (PFS) in R/M SCCHN treated with various systemic treatments that included platinum-chemotherapy, a single agent anti-PD(L)1 inhibitor, or an immunotherapy combination.

Here we show that early ctDNA kinetics, using an in-house tumour-agnostic assay, predict the efficacy of nivolumab or pembrolizumab monotherapy when administered as standard of care in R/M SCCHN.

2. Materials & methods

2.1. Patient cohort, study design, and inclusion criteria

After obtaining written informed consent, all SCCHN patients were prospectively enroled into UCL-ONCO-2013 (2013/12FEV/047), a non-interventional multicentric biomarker trial collecting whole blood, plasma samples every 2 months and, when feasible, tumour biopsies (NCT02139020). The clinical characteristics and disease outcomes of each patient were prospectively recorded in a secured database (REDCap) [12]. The study was approved by our ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000).

Eligibility criteria were: patients (i) enroled 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 non-curative intent anti-PD1 therapy without concomitant chemotherapy according to standard of care, (iii) for whom whole blood as well as pre-treatment and first on-treatment plasma samples were available, and (IV) treated for at least 6 weeks with anti-PD1 therapy to match the treatment resistance definition proposed by Society for Immunotherapy of Cancer that requested at least 6 weeks of immunotherapy exposition. [13] The first on-treatment plasma sample to calculate ctDNA kinetics (ΔctDNA), defined as FU1, had to be collected within 6–10 weeks after the initiation of the PD1 inhibitor. Nasopharyngeal, salivary, and sinonasal cancers were excluded. HPV-driven oropharyngeal cancers were defined as tumours that were positive for HPV by in-situ hybridisation.

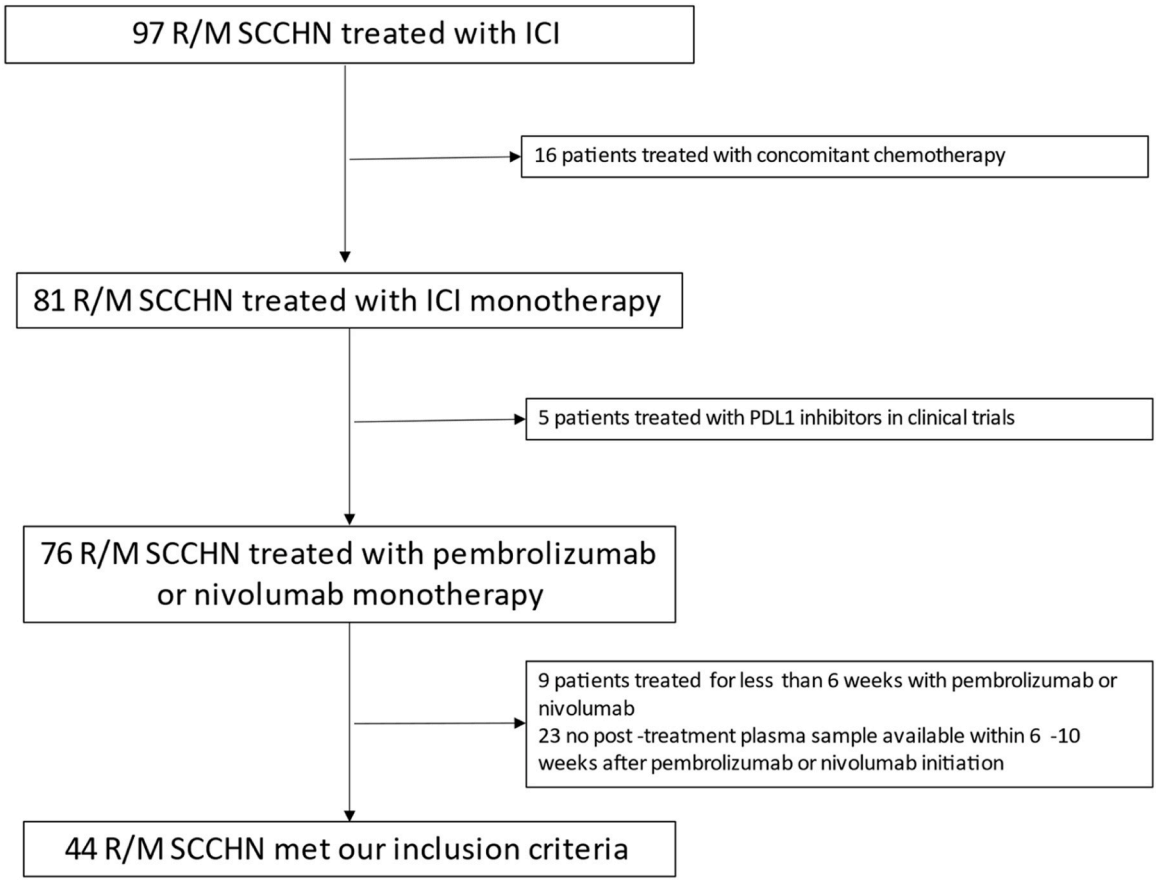

Fig. 1. Patient workflow. ICI: immune checkpoint inhibitor; R/M SCCHN: Recurrent and/or metastatic squamous cell carcinoma of the head and neck.

2.2. Cell free DNA (cfDNA) targeted next-generation sequencing (NGS) and variant calling

NGS was performed after capture using a custom-made in-house panel of 37 genes plus two HPV16 oncogenes (E6 and E7) designed by Twist Bioscience®, with an expected mean coverage of 2000x. The 37 genes were selected because they are frequently mutated in HPV-negative R/M SCCHN [14,15]. We added the following genes due to their possible implication in immune response: *BRCA2*, *JAK2,* and *B2M*. The list of genes can be found in Appendix III-Table 1.

All pre-treatment plasma samples were first analysed with Kraken2 (v. 2.1.2, database "Standard", March 2023), [16] a taxonomic sequence classifier that assigns taxonomic labels to DNA sequences. A patient was considered HPV16-positive if at least one read was assigned to the HPV16 genome in the pre-treatment plasma sample, according to Kraken2. Otherwise, the patient was considered HPV16-negative.

2.3. Study objective and endpoints

Our main objective was to develop a tumour-agnostic assay to evaluate ctDNA kinetics as a biomarker of response to therapy in HPV-negative and -positive R/M SCCHN. Our primary endpoint was the concordance between best overall response (BOR) according to Response Evaluation Criteria in Solid Tumors version 1.1 (RECISTv1.1) [17] and ΔctDNA. Secondary endpoints were PFS, OS, and cancer-specific survival (CSS) in favourable and unfavourable ΔctDNA in patients who had ctDNA detected in their pre-treatment plasma sample. As an exploratory analysis, we investigated if alterations in genes implicated in certain cancer-related pathways in the pre-treatment sample were associated with disease outcome.

ΔctDNA was defined for HPV16-negative patients as the difference in mean variant allele frequency (VAF) of all variants between the pre-treatment sample and the FU1 sample harvested 6–10 weeks after PD1 inhibitor initiation, as described previously (ΔctDNA = mean FU1 VAF - mean pre-treatment VAF). [11] For HPV16-positive patients, we defined ΔctDNA as the difference in the number of reads assigned to the HPV16 genome by Kraken2 [16] between the pre-treatment sample and the FU1-sample (ΔctDNA = FU1 HPV16 reads - pre-treatment HPV16 reads). Therefore, positive ΔctDNA means there was an increase in ctDNA in the FU1 sample compared with the pre-treatment sample, and negative ΔctDNA means there was a decrease in ctDNA in the FU1 compared with the pre-treatment sample.

2.4. Statistics

Concordance between ΔctDNA and BOR in patients was evaluated using a Chi-square test. We hypothesised that patients with negative ΔctDNA will achieve as BOR either a complete response (CR), partial response (PR), or stable disease (SD), whereas patients with positive ΔctDNA will experience progressive disease (PD) as BOR. We compared the proportion of patients who fulfilled these criteria against those who did not.

PFS, OS, and CSS were estimated using Kaplan-Meier methods. Patients without an event (progression or death) were censored at the date of last follow-up. Differences in PFS and OS between patients with negative and positive ΔctDNA were assessed using the log-rank test. A p-value <0.05 was considered to be statistically significant.

All analyses were done using R (v4.2.1).

Methodology for blood and plasma collections, cell free DNA (cfDNA) and germline extraction, cfDNA NGS, variant calling, and imagery evaluation can be found in Appendix I- Material & Methods 1.

Table 1
Patient characteristics.

	Overall
	(N = 44)
Sex	
Male	33 (75.0%)
Female	11 (25.0%)
Age (years)	
Median [Min,Max]	68.5 [44.0, 79.0]
Primary location	
Oral cavity	13 (29.5%)
Oropharynx	17 (38.6%)
P16 status	
Negative	10 (22.7%)
Positive	7 (15.9%)
HPV-16 ISH	
Negative	4 (9.0%)
Positive	2 (4.5%)
Missing /not enough material	1 (2.3%)
Hypopharynx	4 (9.0%)
Larynx	3 (6.8%)
Head and Neck unknown primary	6 (13.6%)
Two locations (oropharynx and oral cavity)	1 (2.3%)
Previous curative-intent treatment regimen	
Primary chemoradiation	15 (34.1%)
Primary radiation	7 (15.9%)
Primary surgery +/- (chemo)radiation	13 (29.5%)
No previous curative treatment	9 (20.5%)
CPS Score	
<1	7 (15.9%)
1–19	16 (36.4%)
≥20	19 (43.2%)
Not enough material	2 (4.5%)
Tobacco consumption (>10PY)	
Yes	32 (72.7%)
No	12 (27.3%)
Alcohol consumption (>2U/day)	
Yes	30 (68.2%)
No	14 (31.8%)
PD1 Inhibitor	
Nivolumab	24 (54.5%)
Pembrolizumab	20 (45.5%)
R/M line in which immunotherapy was administered	
First-line	28 (63.6%)
Second-line	14 (31.8%)
Third-line	2 (4.5%)
Previously treated with platinum agents	
No	13 (29.5%)
Yes	31 (70.5%)
Disease location before the start of Immunotherapy	
Loco-regional	37 (84.1%)
Loco-regional & metastatic	7 (15.9%)
Metastatic only	0 (0.0%)

R/M: recurrent and/or metastatic, HPV16: Human Papilloma Virus 16, ISH: In-situ hybridisation, CPS: combined positive score, PY: packet/year, U: units

3. Results

3.1. Patient characteristics

Between June 2017 and July 2022, 97 R/M SCCHN patients treated with an immune checkpoint inhibitor (ICI) were enroled in the UCL-ONCO-2013 biomarker trial. Forty-four patients met the inclusion criteria (Fig. 1). Patient clinical characteristics are described in Table 1 and in Appendix II-Fig. A. The median OS for the 44 patients was 14.4 [95% confidence interval (CI): 8.6–26.8] months and the median PFS was 2.9 [95% CI: 2.4–7.2] months. Patients with combined positive score (CPS) ≥1 had a longer OS (median OS 16.6 [95% CI: 12.5–41.5] months compared to patients with CPS < 1 (median OS 7.2 [95% CI: 6.6-not evaluable (NE)] months); p = 0.027), but similar PFS (median PFS: 2.9 [95% CI: 2.4–11.9] months and 6.6 [95% CI: 1.7–7.2] months, respectively; p = 0.38). No significant differences were observed between patients treated with pembrolizumab or nivolumab for OS ((Hazard Ratio)HR=0.92, [95% CI: 0.43–1.97]) or for PFS (HR=0.85,

Table 2
Best overall response and ΔctDNA.

RECISTv1.1	Negative ΔctDNA	Positive ΔctDNA	Total
CR	2 (5.7%)	1 (2.9%)	3 (8.6%)
PR	5 (14.3%)	1 (2.9%)	6 (17.1%)
SD	6 (17.1%)	3 (8.6%)	9 (25.7%)
PD	4 (11.4%)	13 (37.1%)	17 (48.6%)
Total	17 (48.6%)	18 (51.4%)	35 (100%)

CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, RECISTv1.1: Response Evaluation Criteria in Solid Tumours version 1.1

[95% CI: 0.44–1.68]). Similarly, there was no significant difference between patients treated with ICI monotherapy in the first, second, or third line of treatment (OS: HR=0.83 [95% CI: 0.38–1.79]; PFS: HR=0.74 [95% CI: 0.38–1.45]).

One hundred and forty-seven plasma samples were analysed among these 44 patients (Appendix II Fig. A).

3.2. Tumour-agnostic panel detects pre-treatment ctDNA

Pre-treatment ctDNA was detected in 35 (80%) out of the 44 patients. HPV16 DNA was detected in three patients in the pre-treatment samples using Kraken2. The number of reads in the pre-treatment samples assigned to the HPV16 genome was 2459, 32, and 7 in each patient, respectively. Among the 41 HPV16-negative patients, ctDNA was detected in 32 patients (78%) in the pre-treatment samples. The most frequently mutated genes were *TP53* (32%), *SPEN* (18%), *EP300* (18%), *SMG1* (15.6%), and *KMT2C* (15.6%). Median VAF was 1.3% [range: 0.26–31.0%].

3.3. ΔctDNA is correlated with BOR

Among the 35 patients with ctDNA in their pre-treatment samples, 17 and 18 patients had a decrease (negative ΔctDNA) and an increase (positive ΔctDNA) in ctDNA mean VAF, respectively.

In the overall cohort, the overall objective response rate was 25.7% (9/35) (CR: 3/35 (8.6%); PR: 6/35 (17.1%)). SD and PD as BOR were achieved in 9 (25.7%) and 17 (48.6%) patients, respectively. Among these 35 patients, no patients had pseudo-progression, defined as initial PD followed by partial or complete response.

Among the 17 patients with negative ΔctDNA, 13 (76.4%) achieved either a CR, PR, or SD, and 4 (23.5%) had PD. Thirteen (72%) out of 18 with a positive ΔctDNA had PD as BOR (Table 2).

ΔctDNA correctly predicted the BOR according to RECISTv1.1 in 26 (74%) out of 35 patients, whereas 9 (26%) patients had imaging and ΔctDNA discordant results ($X^2 = 4.51$; $p = 0.033$).The median time

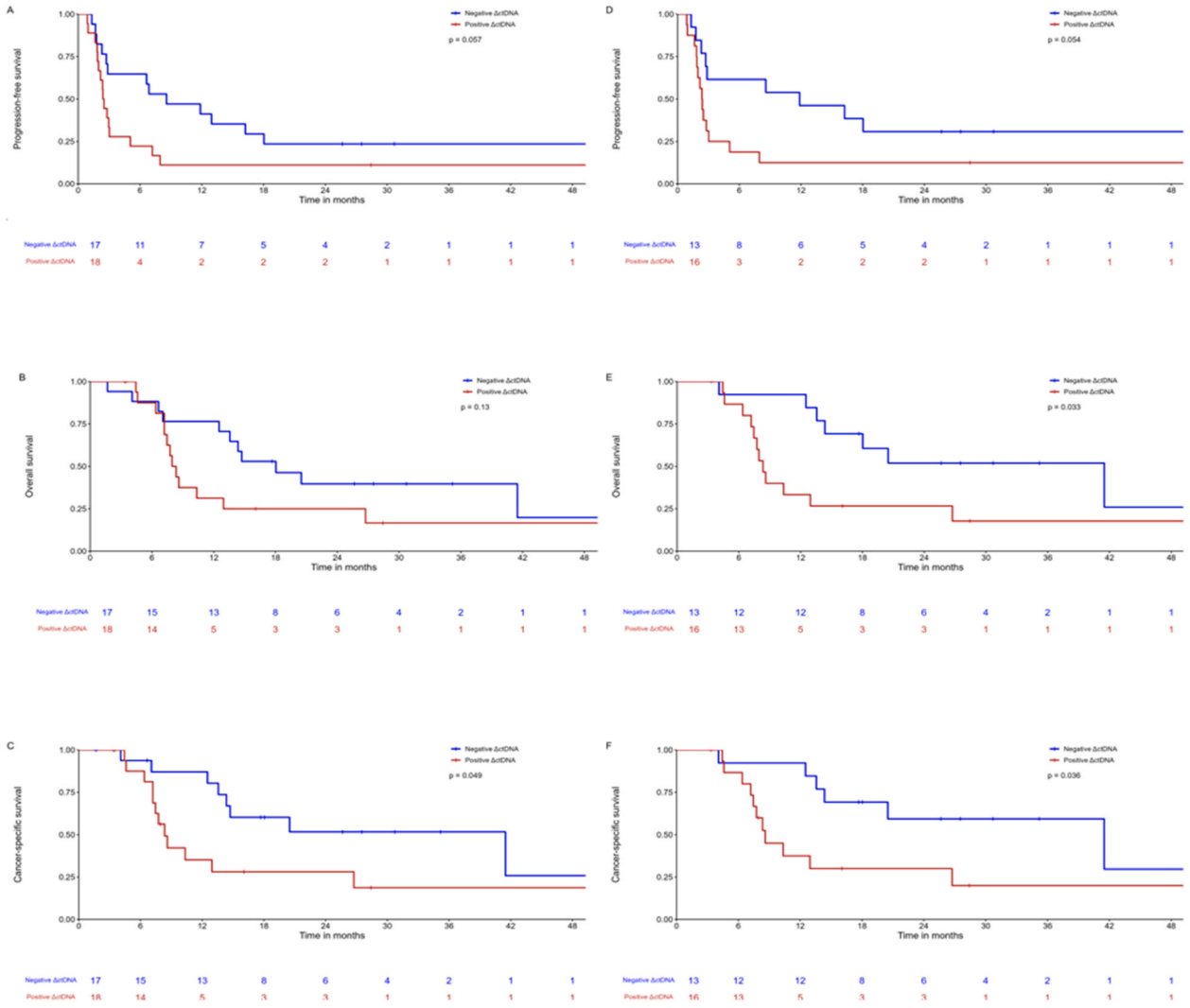

Fig. 2. Kaplan-Meier estimate of progression-free survival (PFS) (Panels A), overall survival (OS) (Panels B), and cancer-specific survival (Panels C) for positive and negative ΔctDNA patients in the whole population. Kaplan-Meier estimate of PFS (Panels D), OS (Panels E), and cancer-specific survival (Panels F) for positive and negative ΔctDNA patients in PD-L1 CPS >1 population. Blue curves = negative ΔctDNA patients; red curves = positive ΔctDNA patients.

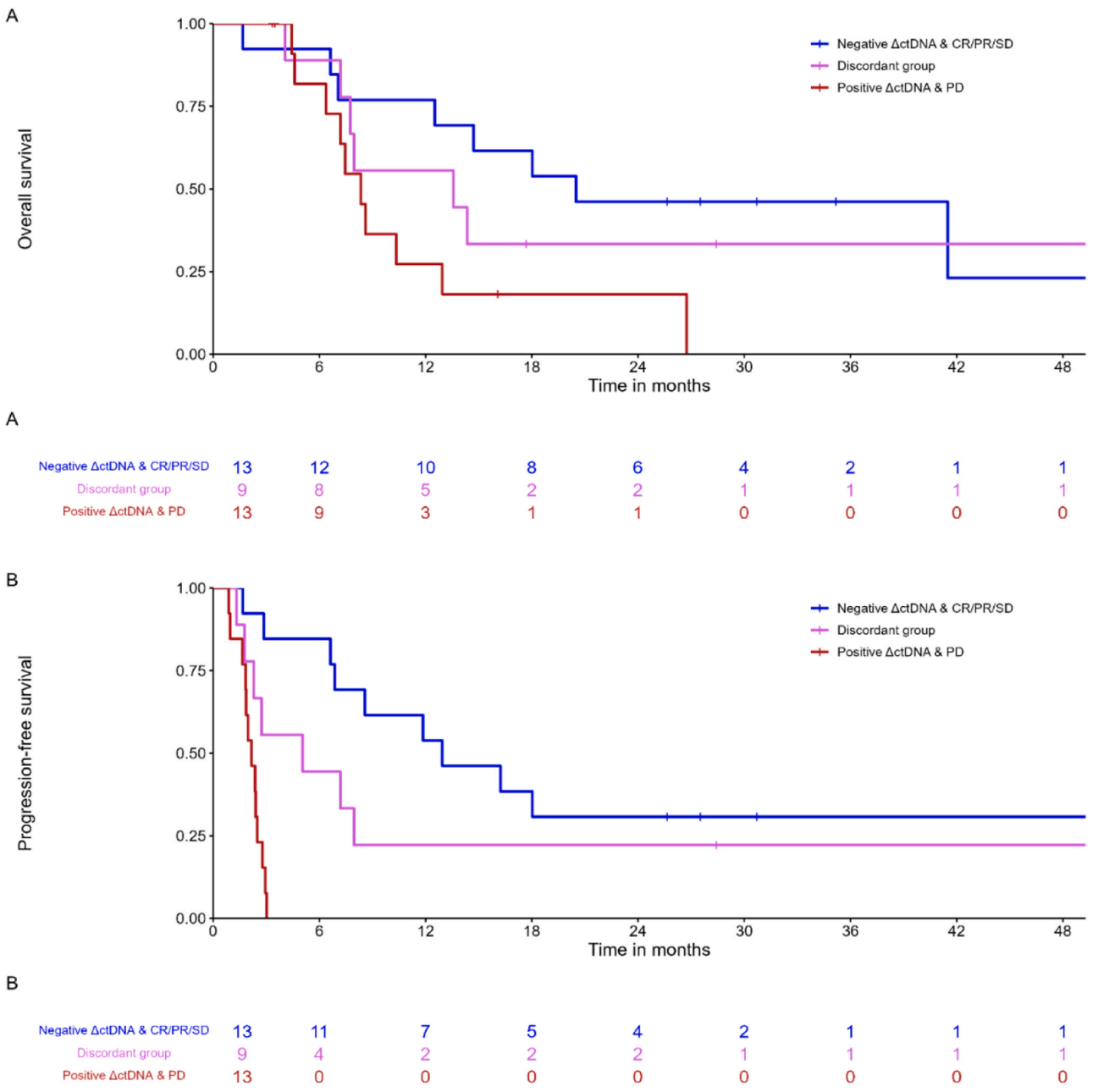

Fig. 3. Kaplan-Meier estimate of OS (A) and PFS (B) for negative ΔctDNA & CR, PR and SD patients, positive ΔctDNA & PD patients, and discordant patients. Blue curves = negative ΔctDNA negative & CR, PR, and SD; red curves = positive ΔctDNA patients & PD; pink curves = discordant patients.

between the increase in ctDNA and the time of PD or death was 30 days (range: 3–347). The median time between the increase in ctDNA and the time when immunotherapy was stopped was 138 days (range: 11–1079). The mean and median lead-time between the first on-treatment ctDNA assessment (FU1) and the first disease evaluation by imagery was 13.6 weeks (95.4 days) and 4.3 weeks (30 days), respectively (range from 3 to 434 days).

3.4. ΔctDNA to predict PFS, OS, and CSS

Median PFS was 8.6 [95% CI: 2.73–18.1] months in the negative ΔctDNA group and 2.5 [95% CI: 2.0–3.0] months in the positive ΔctDNA group ($p = 0.057$, Fig. 2A). Median OS was 18.1 [95% CI 12.5–41.5] and 8.2 [95% CI 7.2–13.0] months in the negative and positive ΔctDNA groups, respectively ($p = 0.13$) (Fig. 2B). As six patients died from causes other than their cancer (2 from pneumonia, 1 from hip fracture, 1 from sudden death, 1 from Covid infection, and 1 from cerebrovascular stroke), we calculated the CSS. CSS was significantly better in the negative ΔctDNA group than in the positive ΔctDNA group: median CSS 41.5 [95% CI: 14.4-NE] and 8.35 [95% CI: 7.2–26.8] months, respectively ($p = 0.049$) (Fig. 2C).

3.5. ΔctDNA predictive value in different subgroups

In patients with PD-L1 expressing SCCHN (CPS ≥1), there were significant differences between patients with positive and negative ΔctDNA for OS (median OS: 8.4 [95% CI: 7.5–13.0] and 41.5 [95% CI: 14.4-NE] months; $p = 0.033$), and CSS (8.6 [95% CI: 7.526.8] months and median OS: 41.5 [95% CI: 14.4-NE]; $p = 0.036$), but not for PFS (median PFS: 2.4 [95% CI: 1.9–3.03] months and 11.9 [95% CI: 2.7-NE]; $p = 0.054$), (Fig. 2 D-F).

Some patients had discordant results between ΔctDNA and imaging

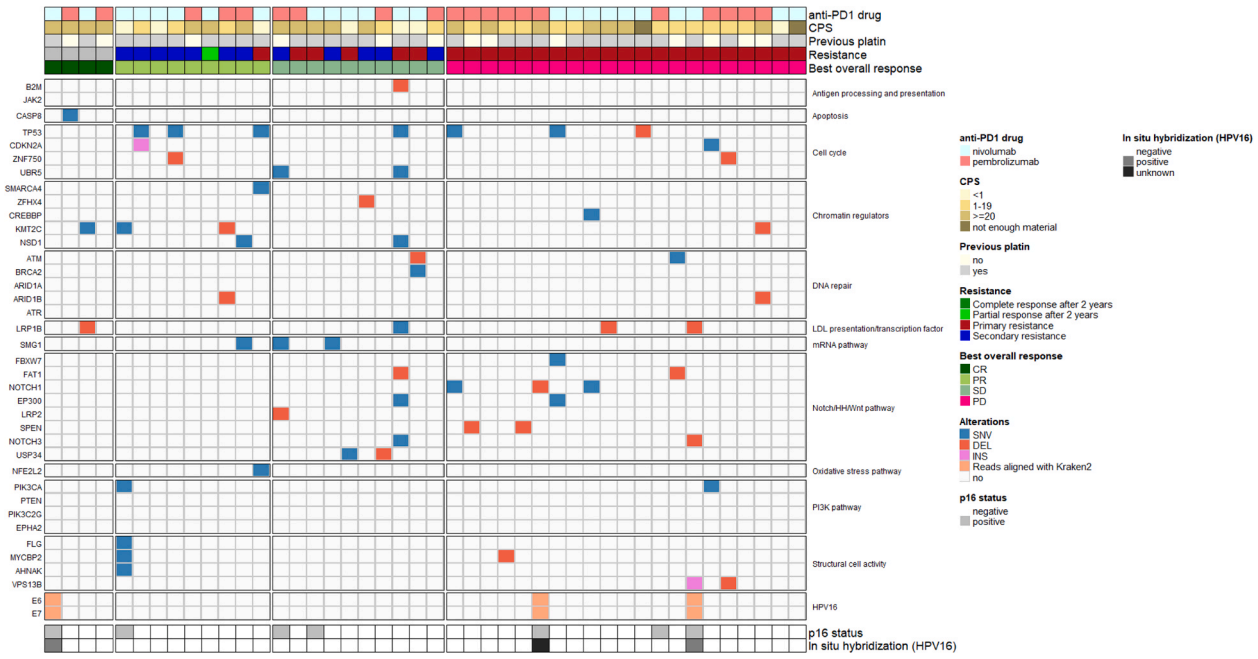

Fig. 4. Mutations in pre-treatment plasma sample and patient characteristics. PD1: Programmed cell death 1, CPS: combined positive score, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, SNV: single nucleotide variation, DEL: deletion, INS: insertion.

response. Four patients had a negative ΔctDNA with disease progression, whereas five patients had a positive ΔctDNA with either a CR, PR, or SD at first imaging evaluation. Interestingly, we observed that these discordant patients (n = 9) had an intermediate prognosis. For the discordant group, the median OS was 13.6 [95% CI: 7.8-NE] months compared with 20.5 [95% CI: 15.5-NE] months for the patients with negative ΔctDNA and either a CR, PR, or SD (p = 0.5) and 8.4 [95% CI: 6.4–13] months for the patients with positive ΔctDNA and PD (p = 0.2). Similarly, median PFS of the discordant group was 5.1 [95% CI: 2.3–8.0] months compared with 13.0 [95% CI: 6.9-NE] (p = 0.2) and 2.2 [95% CI: 1.8–2.5] months (p < 0.001), respectively (Fig. 3).

3.6. Prognostic value of pre and on-treatment mutations

The mutations considered to be damaging and found in the pre-treatment plasma samples are depicted in Fig. 4. Interestingly, we observed that inactivating mutations in genes implicated in the Notch/Hedgehog/Wnt pathways were only identified in patients with SD or PD as BOR and not in patients who had a CR or PR. There was a significant difference in PFS between patients with pre-treatment plasma harbouring mutations in genes implicated in the Notch/Hedgehog/Wnt pathways compared to the others: median 2.07 [95%IC: 1.8–2.7] versus 6.8 [95%IC: 2.9–13.0] months (p = 0.024). There was, however, no statistical difference in OS: 8.2 [95%IC: 6.6–13.6] versus 14.7 [95% IC: 8.6–41.5] months (p = 0.083)(Appendix II Figs. B & C).

Another patient with primary resistance had a pre-treatment ctDNA *B2M* mutation. In contrast, in patients with long-term CR, we observed one patient with a *CASP8 (*p.E123Q*)* mutation and another with *KMT2C* (p.C1150Y) and *LRP1B* (p.G683fs) mutations.

However, although ctDNA increased in most of the patients at disease progression, we could not link any damaging mutations with primary or secondary resistance (Appendix II Fig. D).

4. Discussion

In our study, concordance between ctDNA kinetics and imaging response was observed in 26 (74%) out of 35 patients. ctDNA kinetics also identified a group of R/M SCCHN patients who had a better outcome when treated with nivolumab or pembrolizumab monotherapy and uncovered patients less likely to benefit from a PD1 inhibitor who might benefit from the rapid addition of chemotherapy or a change of therapy. To our knowledge, our series is the largest R/M SCCHN cohort treated with anti-PD1 monotherapy.

Today, anti-PD1 therapy is mainly prescribed for patients with R/M SCCHN who express PD-L1. We therefore conducted a subgroup analysis in this setting and confirmed that ctDNA kinetics also predicted disease outcome in R/M SCCH patients expressing PD-L1 (Fig. 3D-F). Interestingly, in our trial, patients with a CPS >1 and negative ΔctDNA had a median survival of 41.5 months, suggesting that combining these molecular biomarkers should be further investigated in a larger group of patients.

Some patients had discordant results between ΔctDNA and imaging response. We observed that these discordant patients had an intermediate prognosis compared with patients with non-discordant results. The discordant results may be partly explained by the patients with small variations in ctDNA quantity between pre- and on-treatment plasma samples. Similarly, Bratman et al. [9] also showed that ctDNA dynamics, when associated with imaging evaluation at 2–3 months, was also better at discriminating between poor and good prognosis patients in several cancer types.

Evaluation of ctDNA kinetics at 6–10 weeks is close to the first imagery assessment and further studies should investigate if earlier times are also clinically relevant. However, other groups have suggested that ctDNA can be useful to discriminate real progression from pseudo-progression. [18–20] In our study, no patients experienced pseudo-progression. We used a tumour-agnostic methodology that could be easily implemented in daily practice as it does not require a tumour biopsy. Although tumour-informed personalised methodologies have been shown to have a good ctDNA detection rate, [21] tumour sequencing might be problematic as lesions at progression might not be biopsied for safety reasons, and R/M SCCHN may have a different genomic landscape than primary tumours. [15] Additionally, quality issues with the tumour biopsy can also preclude confident variant analyses, particularly in the R/M setting. [22] However, our small in-house gene panel detected ctDNA in only 80% of patients prior to treatment initiation. According to results from Taylor et al. [11] a broader

squamous cell-specific panel using CAPP-Seq may have a better ctDNA detection rate.

Although limited by the low number of genes in our panel and the small number of patients, we observed that some pre-treatment damaging ctDNA mutations seemed to be linked to disease outcome. The presence of mutations in *CASP8,* [23,24] *LRP1B,* [25,26] and *KMTC2* [27,28] has already been described as a possible biomarker of favourable outcome in patients undergoing immunotherapy in other studies. [23,29] Conversely, *B2M* is required for effective antigen presentation, and this specific mutation has been previously described to predict anti-PD1 therapy resistance. [30] The Notch/Hedgehog/Wnt pathways may also be implicated in immunotherapy resistance or poor prognosis in SCCHN. [31,32] However, the on-treatment plasma samples did not reveal any specific pattern of ctDNA mutations associated with disease progression or response, highlighting the complexity of treatment resistance mechanisms.

5. Conclusion

Tumour-agnostic ctDNA analysis for HPV-negative and HPV-positive R/M SCCHN is feasible. ctDNA dynamics show promising results in predicting response to immunotherapy in R/M SCCHN. Further investigations are needed to integrate ctDNA kinetics with other prognostic and predictive biomarkers to better predict treatment and cancer outcomes.

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CRediT authorship contribution statement

N.H. and J-P.M are co-corresponding authors and each has significant contribution to this work. Concept and design: N.H., A.V.D.E., and J-P.M. Acquisition, analysis, or interpretation of data: N.H., A.V.D.E., A. D., C.V.M., R.G., and J-P.M. Drafting of the manuscript: N.H., and J-P.M. Critical revision of the manuscript for important intellectual content: All authors. Final approval of completed version of manuscript: All authors. Bioinformatics and statistical analysis: N.H., A.V.D.E., C.V.M, R.H., and J-P.M. Administrative, technical, or material support: N.H., A.M., and H. D. Supervision: J-P.M. Accountability for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: All authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Disclosure

The authors declare no potential conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.113372.

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