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Original Research

Circulating tumour DNA kinetics in recurrent/metastatic head and neck squamous cell cancer patients

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Abstract ***Purpose:*** Immune checkpoint blockade (ICB) has become a standard of care in the treatment of recurrent/metastatic head and neck squamous cell cancer (R/M HNSCC). However, only a subset of patients benefit from treatment. Quantification of plasma circulating tumour DNA (ctDNA) levels and on-treatment kinetics may permit real-time assessment of disease burden under selective pressures of treatment.

Patients and methods: R/M HNSCC patients treated with systemic therapy, platinum-based chemotherapy (CT) or ICB, underwent serial liquid biopsy sampling. Biomarkers tested included ctDNA measured by CAncer Personalized Profiling by deep Sequencing (CAPP-Seq) and markers of host inflammation measured by neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR).

Results: Among 53 eligible patients, 16 (30%) received CT, 30 (57%) ICB [anti-PD1/L1] monotherapy and 7 (13%) combination immunotherapy (IO). Median progression-free survival (PFS) and overall survival (OS) were 2.8 months (95% CI, 1.3–4.3) and 8.2 months (95% CI, 5.6–10.8), respectively. Seven (13%) patients experienced a partial response and 21 (40%) derived clinical benefit. At baseline, median ctDNA variant allele frequency (VAF) was 4.3%. Baseline ctDNA abundance was not associated with OS (p = 0.56) nor PFS (p = 0.54).

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However, a change in ctDNA VAF after one cycle of treatment (ΔVAF (T1–2)) was predictive of both PFS ($p < 0.01$) and OS ($p < 0.01$). Additionally, decrease in ΔVAF identified patients with longer OS despite early radiological progression, 8.2 vs 4.6 months, hazard ratio 0.44 (95% CI, 0.19–0.87) $p = 0.03$. After incorporating NLR and PLR into multivariable Cox models, ctDNA ΔVAF retained an association with OS.

Conclusions: Early dynamic changes in ctDNA abundance, after one cycle of treatment, compared to baseline predicted both OS and PFS in R/M HNSCC patients on systemic therapy.

1. Introduction

Immuno-oncology agents have become a standard-of-care in the treatment of recurrent/metastatic head and neck squamous cell cancer (R/M HNSCC), initially in the second-line setting in a programmed death ligand-1 (PD-L1) unselected population [1,2] and subsequently in the first-line setting stratified by PD-L1 status, alone or in combination with chemotherapy (CT) [3]. However, only a subset of patients benefits from such treatment with a durable clinical response.

Currently, there are no validated biomarkers beyond that of PD-L1 status, which in itself has limitations, that aid in the prediction of response to immune checkpoint inhibitors in R/M HNSCC. Circulating tumour DNA (ctDNA) within peripheral blood plasma provides non-invasive access to cancer-specific somatic mutations [4]. Highly sensitive quantification of plasma ctDNA and its kinetics may permit real-time assessment of disease under selective pressures of treatment. Recent studies have demonstrated feasibility for the detection of molecular residual disease prior to clinical recurrence in locally advanced HNSCC; and broad clinical validity in the monitoring of response to anti-PD1 antibody in pan-cancer patients with recurrent/metastatic disease [5–7].

In addition to ctDNA, peripheral blood provides access to circulating markers of host inflammation, such as the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR), which have shown prognostic ability across cancer types and stages of disease [8–10]. These metrics are easily derived from standard pre-treatment blood tests and provide readily available information with the potential to aid clinical decision-making.

Here we present the results of a prospective observational study characterising ctDNA dynamics under the treatment selection pressure of systemic therapy, correlating with clinical outcome in R/M HNSCC patients.

2. Patients and methods

2.1. Patients and study design

From 16 May 2018 to 05 December 2019, 55 patients with R/M HNSCC were enroled in Multi-omic Assessment of Squamous cell cancers receiving Systemic Therapy (MASST) (NCT03712566). The study was approved by the Research Ethics Board at Princess Margaret Cancer Centre, University Health Network; all patients provided written informed consent. Treatment was delivered at Princess Margaret Cancer Centre: patients received either (a) first line platinum-based CT, (b) immune checkpoint blockade (ICB) [anti-PD1/L1] monotherapy as per standard of care, second line in a PD-L1 unselected population, or (c) combination IO, as part of a therapeutic clinical trial. Of note, at the time that this study was active, first-line ICB alone or in combination with CT was not available in Canada as a standard of care; therefore, PD-L1 status according to combined positive score was not routinely tested for these patients. The MASST study has completed enrolment but remains open for continued follow-up of patients. The data collection cut-off date was 08 September 2022.

2.2. Blood collection and processing

Peripheral blood plasma was collected as part of an institutional liquid biopsy collection study, Liquid Biopsy Evaluation and Repository Development at Princess Margaret (LIBERATE) (NCT03702309). Samples were collected before cycles 1, 2 and 3 (each cycle is every 3 or 4 weeks depending on treatment regime), and at the time of disease progression, corresponding to timepoints (T) 1–4. T1 was considered baseline, prior to treatment initiation. At each collection time point, 30 ml of peripheral blood was collected in Cell-Free DNA (cfDNA) BCT RUO tubes (Streck, La Vista, NE). Plasma was separated from the cell pellet within 2 h of collection and aliquoted for storage at −80 °C. cfDNA was purified from clarified plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). Peripheral blood leucocyte (PBL) genomic DNA was extracted using the AllPrep DNA/RNA/miRNA Universal Extraction Kit (Qiagen). All cfDNA samples were collected and processed by the Translational Genomics Programme at the Ontario Institute for Cancer Research (Toronto, Canada).

2.3. CAncer Personalized Profiling by deep Sequencing methods

2.3.1. Sequencing and data preprocessing

To detect and longitudinally monitor ctDNA at available time points, CAncer Personalized Profiling by deep Sequencing (CAPP-seq) was conducted on Illumina-compatible libraries using a hybrid capture panel optimised for squamous cell malignancies, as previously published [11,12]. Libraries were constructed from 10 to 30 ng of cfDNA or sheared PBL genomic DNA. The 480 kb CAPP-seq panel of biotinylated bates (Integrated DNA Technologies (IDT), Coralville, IA) covers exons from 580 squamous cancer-related genes. To suppress sequencing errors, duplex sequencing was conducted using unique molecular identifiers that were built into the indexed adaptors. Reads were aligned to the human reference genome hg19 using Burrows-Wheeler Aligner (BWA) (v0.7.15), and base quality score recalibration was conducted using the Genome Analysis ToolKit (GATK) Base Quality Score Recalibration (BQSR) (v 3.8) according to GATK best practices [13]. The median sequencing depth for tumour and matched-normal samples were 6483x and 880x, respectively. The aligned reads were sorted and indexed on the genome positions using SAMtools (v 1.9) [14]. ConsensusCruncher was used to generate consensus sequences on the aligned reads [15]. Duplex consensus sequences were used for the subsequent variant calling, and all unique molecule sequences were used for the evaluation of variant allele frequency (VAF).

2.3.2. Variation calling

Mutect2 was used to identify both somatic and small insertions/deletions (Indels) on a joint calling of multiple tumour samples and a matched normal sample for each patient [16]. Putative germline mutations with VAF > 25% across all tumour samples were removed, and functional mutations were selected. To suppress false Indel calls, we took the intersection of those called by both Mutect2 and VarDict2 [17] and required at least five supporting reads. Somatic mutations called on baseline samples (i.e. T1) were used for the subsequent analyses, and the median VAFs of the selected mutations were summarised to represent the ctDNA abundance.

2.4. Peripheral blood parameters

NLR and PLR, circulating markers of host inflammation, were obtained from standard-of-care bloodwork at baseline (T1) and following one cycle of treatment (T2); dichotomised by median values and correlated with clinic-pathological features and survival.

2.5. Statistical analysis

Descriptive statistics were used to summarise patient and clinical characteristics, with median and range for continuous variables and frequency and percentage for categorical variables. Survival was performed using the Kaplan-Meier method and the log-rank test by the lifelines library [18]. The Cox proportional hazards model was used to relate variables with survival [18]. Overall survival (OS) was defined as time from first treatment to the date of death or the last date of follow up. Progression-free survival (PFS) was defined as time from first treatment to the date of progression, death or last follow-up, whichever occurred first. Progression date was defined as the date of disease progression based on physicians' assessment by Response Evaluation Criteria in Solid Tumours, version 1.1 (RECIST v1.1) [19] or the date of clinical progression if the patient discontinued treatment due to clinical deterioration.

3. Results

Among the 55 patients enroled, 53 (96%) were included in the analysis having received one or more cycles of treatment. Patients received either (a) first line (1L) platinum-based CT (n = 16) as per standard of care, (b) second line (2L) ICB [anti-PD1/L1] monotherapy (n = 30) in a PD-L1 unselected population, as per standard of care, or (c) combination IO with two agents (n = 7) as part of a therapeutic clinical trial, in second

Table 1
Patient characteristics.

		Treated pts N = 53 (%)
Median Age – years (range)		62 (20–82)
Gender	Male	40 (75)
	Female	13 (25)
ECOG PS	0	4 (8)
	1	49 (92)
Smoking History	Current/Previous >15 PYH	36 (67)
	Previous <15 PYH	4 (8)
	Never	13 (25)
Primary Site	Oral Cavity	13 (25)
	Oropharynx	26 (49)
	Larynx	8 (15)
	Hypopharynx	5 (9)
	Nasal Cavity	1 (2)
human papilloma virus (HPV) status	Positive	15 (28)
	Negative	37 (70)
	Unknown	1 (2)
No. of metastatic sites	1–2	42 (79)
	3+	11 (21)
Treatment	Chemotherapy	16 (30)
	ICB Monotherapy	30 (57)
	Combination IO	7 (13)

ECOG PS, Eastern Cooperative Oncology Group Performance Status; PYH, Pack year history; pts, Patients; ICB, Immune checkpoint blockade; IO, Immunotherapy treatment.

line or beyond (2L+) settings. Patients had a median age of 62, most were male (75%) and were current or former smokers (75%), patient characteristics are summarised in Table 1.

Prior to study enrolment, patients received a median of one previous systemic anti-cancer treatment for their R/M HNSCC (range 0–3). The median number of cycles of current treatment was four (range 1–13), and median follow-up duration was 8.2 months (range 1.8–38.2). Median progression-free survival (PFS) and OS were 2.8 months (95% confidence interval (CI), 1.3–4.3) and 8.2 months (95% CI, 5.6–10.8), respectively. Seven [5 on 2L single-agent ICB and 2 on 1 L CT] patients experienced a partial response (PR), with an objective response rate (ORR: complete or PR) of 13%; and 21 [8 on 2L single-agent ICB and 6 on 1L CT] patients derived clinical benefit (defined as complete response, PR or stable disease (SD) for ≥4 cycles), with a clinical benefit rate of 40%.

3.1. ctDNA levels in R/M HNSCC patients were measured by CAPP-seq

CAPP-seq was applied at each available timepoint, and VAF was calculated and correlated with PFS and OS (Fig. 1). ctDNA was detected in 50/53 (94.3%) patients at baseline, with median VAF of 4.3% (range 0.3–21.8%), Supplementary Fig. 1.

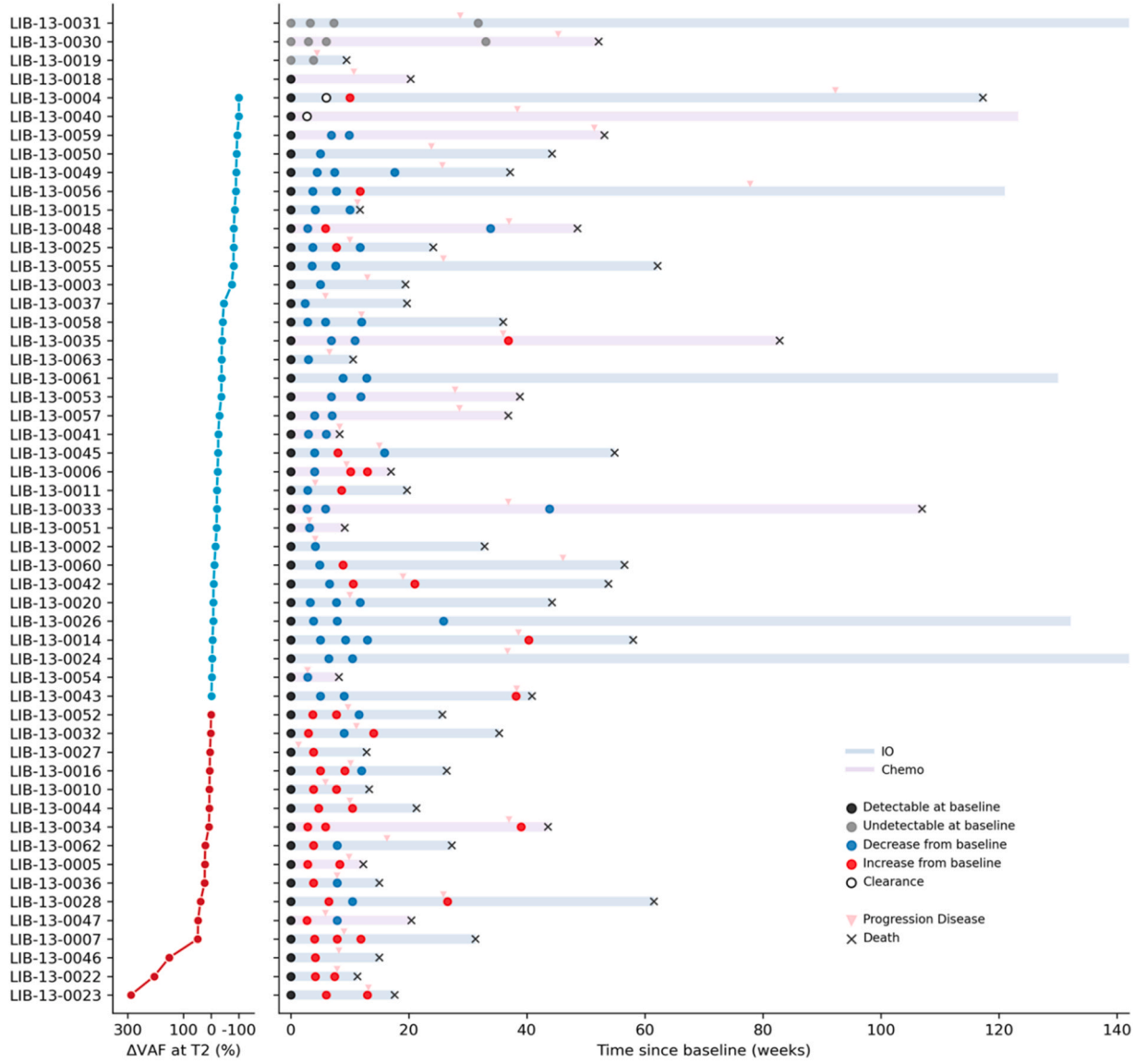


Fig. 1. The change in ctDNA levels from baseline (T1), time to progression of disease and survival status. Fifty-three patients sequenced by CAPP-Seq. Left, patients are ordered according to ctDNA change from T1 to T2 based. The red and blue colours represent ctDNA levels that increased and decreased at T2, respectively. Right, swimmer plot shows clinical outcomes; duration of response, ctDNA detectability and level of change relative to T1 at the other assayed time points, T2–4. Blue represents ICB treated patients and purple chemotherapy treated.

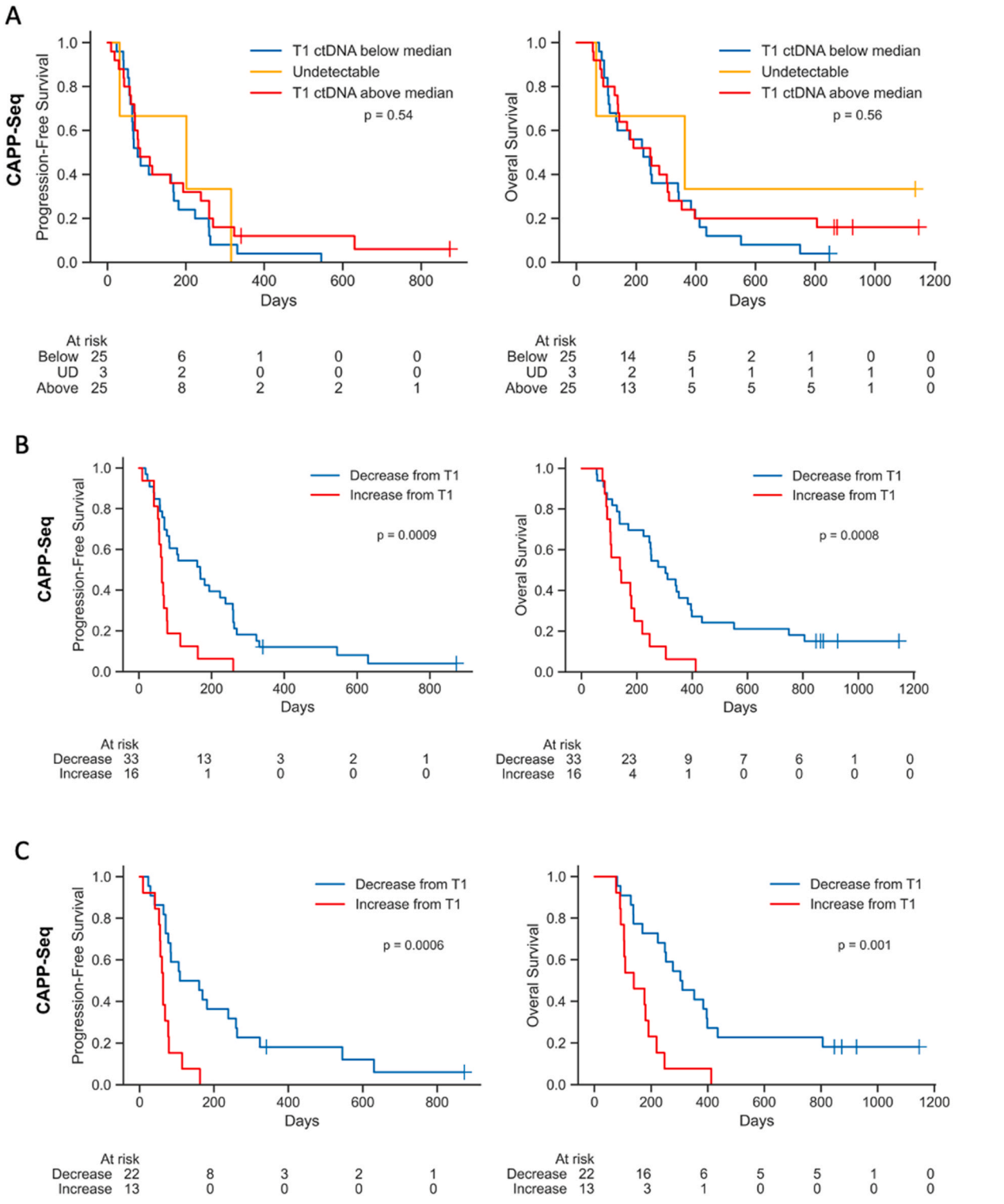


Fig. 2. Association of ctDNA levels at baseline and ctDNA change from T1 to T2 with PFS and OS. (A) Association of ctDNA levels at T1 with PFS and OS. Patients were stratified into groupings according to ctDNA detectability and by the median VAF level (4.3%). Undetectable (UD). Association of changes in ctDNA levels from T1 to T2 with PFS and OS among (B) all patients (n = 49) and (C) IO treated patients (n = 35). Only patients with detectable ctDNA at T1 are included. Increase/decrease is determined from a cut-off of Δ VAF = 0.

3.2. Baseline ctDNA abundance did not correlate with either OS or PFS

Three patients had undetectable ctDNA at baseline. Using the median VAF as a cut off, 4.3%, neither high nor low ctDNA abundance correlated with PFS ($p = 0.54$) or OS ($p = 0.56$), Fig. 2A.

3.3. Change in ctDNA from baseline (T1) to T2, after one cycle of treatment, are predictive of PFS and OS

Early dynamic reductions in ctDNA levels on treatment were predictive of improvement in PFS ($p < 0.01$) and OS ($p < 0.01$) (n = 49) (Fig. 2B). This association was preserved among the subset of patients treated with IO (n = 35) (Fig. 2C).

3.4. Decrease in ctDNA Δ VAF (T1-2) identified patients with longer OS despite early radiological progression

When considering the change in VAF (Δ VAF) in the context of clinical outcomes, those with a decrease in ctDNA between T1 and T2 demonstrated a longer median OS despite radiological response of progressive disease or SD less than 4 cycles, compared to patients with an increase in ctDNA, 8.2 vs 4.6 months, hazard ratio (HR) 0.44 (95% CI, 0.19–0.87, $p = 0.03$) (Table 2). This remained consistent for IO-treated patients, 7.0 vs 4.1 months, HR 0.34 (95% CI, 0.12–0.92, $p = 0.03$) (Supplementary Table 1).

3.5. Baseline peripheral blood NLR and PLR are prognostic of PFS and OS in R/M HNSCC patients receiving systemic therapy

Using median values for NLR and PLR as a cut-off, 7.43 and 450, respectively, baseline NLR and PLR values below median correlated with longer PFS and OS for all patients, Fig. 3. When included in a multivariable Cox model adjusted for age, gender, smoking history and HPV status, a high pre-treatment baseline NLR was a significant predictor of shorter PFS but not OS (Supplementary Table 2). The HR for PFS was 2.9 (95% CI, 1.2–6.8 $p = 0.01$) and HR for OS was 1.7 (95% CI, 0.7–4.0, $p = 0.3$), using the median value of 7.43 to dichotomise patients into two groups. Following one cycle of treatment, a change (T1–2) in NLR or PLR from baseline was not predictive of either PFS or OS.

3.6. Change in ctDNA from baseline (T1) to T2, after one cycle of treatment, remained predictive of OS when incorporated in multivariable analysis

Considering the 49 patients with ctDNA VAF change (T1–2), when included in a multivariable Cox model adjusted for age, gender, smoking history, HPV status, NLR and PLR, a decrease in ΔVAF remained an independent significant predictor of OS, with a HR for OS of 0.4 (95% CI, 0.2–0.9, $p = 0.02$) (Supplementary Table 3).

4. Discussion

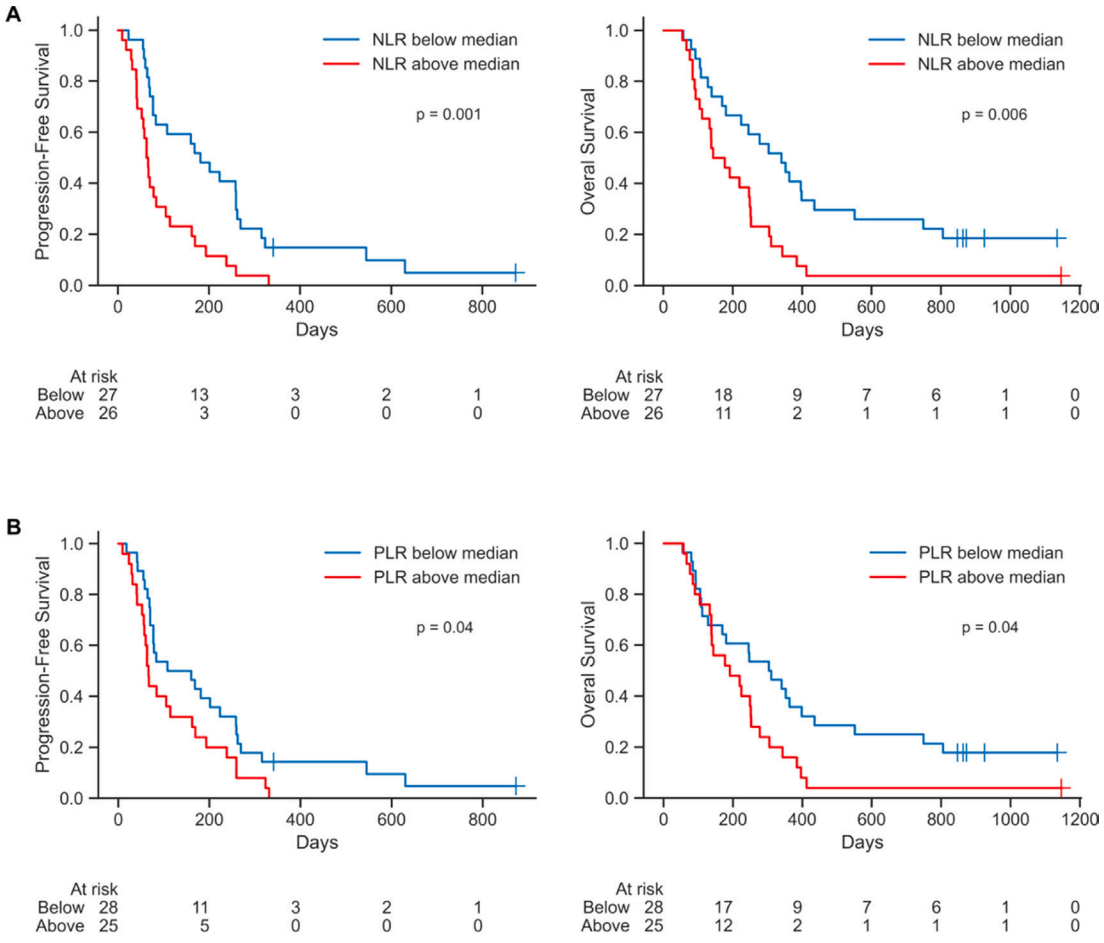
This study prospectively evaluated the longitudinal assessment of ctDNA dynamics under the treatment selection pressure of systemic therapy in R/M HNSCC patients. We utilised a fixed-panel CAPP-seq assay that was optimised for squamous cell cancers [11] to analyse serial plasma samples. Previous studies have shown that ctDNA could be measured in the blood of approximately 70% of patients with metastatic HNSCC assessed by either BEAMing (beads, emulsion, amplification and magnetics) or Safe-Seq (safe-sequencing system) [20–23]; and high levels of ctDNA demonstrated a poor prognostic value and correlated with stage of disease and reduced OS [5].

We found that early dynamic changes in ctDNA, after one cycle of treatment, compared to ctDNA abundance at baseline, were superior to baseline ctDNA values alone as predictors of both PFS and OS. Furthermore, when considering these dynamic changes

Table 2
Decrease in Δ VAF (T1–2) identified patients with longer OS despite early radiological progression.

	Decrease T1-2	Increase T1-2	Survival type: HR (95% CI, *p*-value)
CAncer Personalized Profiling by deep Sequencing			
PR/SD≥ 4cycles	11 [9 IO] (mPFS = 8.6 mo, mOS = 14.5 mo)	1 [1 IO] (mPFS = 5.4 mo, mOS = 13.7 mo)	PFS: 0.22 (0.02, 2.22, $p = 0.19$) OS: 0.61 (0.07, 5.25, $p = 0.65$)
PD/SD < 4cycles	21 [12 IO] (mPFS = 2.8 mo, mOS = 8.2 mo)	15 [12 IO] (mPFS= 2.1 mo, mOS = 4.6 mo)	PFS: 0.45 (0.20, 0.84, $p = 0.03$) OS: 0.44 (0.19, 0.87, $p = 0.03$)

mPFS, Median PFS; mOS, median OS; PR, partial response; SD, stable disease; PD, progressive disease.



	Below median	Above median	Survival type: HR (95% CI; p-value)
All patients			
NLR	**27 [20 IO] (mPFS = 6.0 mo, mOS = 11.3 mo)**	**26 [17 IO] (mPFS = 2.2 mo, mOS = 5.3 mo)**	**PFS: 0.39 (0.22, 0.69; P = 0.001) OS: 0.44 (0.24, 0.80; P=0.006)**
PLR	**28 [19 IO] (mPFS = 4.5 mo, mOS = 10.2 mo)**	**25 [18 IO] (mPFS= 2.2 mo, mOS = 6.4 mo)**	**PFS: 0.56 (0.32, 0.99; P = 0.04) OS: 0.54 (0.30, 0.97; P=0.04)**

Fig. 3. Association of T1 NLR and PLR with PFS and OS. Patients were dichotomised using the median values for NLR and PLR (7.43 and 450, respectively). NLR, Neutrophil to Lymphocyte Ratio; PLR, Platelet to Lymphocyte Ratio.

in a clinical context, a decrease in ΔVAF (T1–2) identified patients with longer OS despite radiological progression at first response imaging. Patients treated with ICB who have radiologically progressed early on in their course of treatment represent a challenging clinical scenario, as a small proportion who are clinically stable and who continue treatment will later derive benefit and extend their survival. In the Checkmate-141 study, of the 60 evaluable patients treated beyond their first RECIST-defined progression, 18 (30%) had a

subsequent reduction in target lesion size, with median OS 12.7 months, compared to 7.7 months in the overall intention-to-treat population [24]. As such, ctDNA in combination with response imaging may play a meaningful role in treatment decision-making for IO-treated HNSCC patients [5].

We used state-of-the-art methods for ctDNA detection and quantification by error-corrected targeted sequencing. The fixed-panel CAPP-seq assay utilised for cDNA detection was tailored to squamous malignancies and includes portion of a large number of genes (n = 580). While in principle this may provide an advantage in advanced disease monitoring by capturing more contemporary alterations and mutations of emerging resistance, the small size of the panel compared with whole exome or genome sequencing may cause certain alterations to be missed. Analysis of tumour tissue or deeper sequencing could also have resulted in refinement of mutation calls [25,26]. Additional pitfalls include the small sample size of this cohort, and although a homogeneous population, there are a number of different therapeutic regimens given at different stages of patients' disease course. Larger studies are needed in R/M HNSCC to determine if and how best ctDNA can be incorporated into the treatment decision paradigm.

Although the changes in longitudinal ctDNA monitoring during treatment could aid in clinical decision-making, liquid biopsies are not yet readily available as routine standard tests in most disease settings. NLR and PLR are simple, inexpensive circulating markers of host inflammation that can be easily interpreted from pre-treatment routine bloodwork. Low NLR and PLR levels at baseline were associated with longer PFS and OS for both CT and ICB-treated patients in our study. Although these blood-based parameters have been studied extensively, there is no definite consensus on the best cut-off to be used [8,27,28]. However, both NLR and PLR are tests that could be easily incorporated into clinical decision-making for prognostication, in addition to current patient fitness parameters such Eastern Co-operative Oncology Group performance status. These blood-based laboratory parameters appear to be most relevant at the baseline pre-treatment time point, suggesting that they most likely denote prognostic biomarkers, in contrast to the predictive value of on-treatment ctDNA dynamics demonstrated in the current MASST study.

Substantial efforts are being made across cancer types to address the challenge of improving the prediction of response to immuno-oncology agents. Currently, no validated predictive biomarkers beyond that of PD-L1 expression have been incorporated into routine practice in R/M HNSCC. Here, we have shown that a change in ctDNA kinetics between baseline and end of the first treatment cycle better predicted both PFS and OS compared to ctDNA abundance at baseline alone. Future studies should seek to integrate ctDNA kinetics with other clinicopathological and radiological parameters. Such multimodal data integration is an important direction for precision oncology and may enhance our ability to identify early predictors of response and resistance to ICB [29,30].

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CRediT authorship contribution statement

K.T., E.E, S.V.B and L.L.S. developed the concept and design of the MASST study. Patient recruitment and sample collection was performed by K.T., M.O., A.S., A.H., and L.L.S. Clinical data collection, analysis and curation was performed by K.T. and M.M. J.Z performed data preprocessing, variant calling, statistical analyses and figure generation. K.T, J.Z and L.L.S wrote the initial manuscript and all authors were involved in the review and finalisation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: KT – Nothing to declare. JZ – Nothing to declare. MM – Nothing to declare. MO reports consultant/advisory role for: Merck, EMD Serono, Transgene; Grant/Research support from (Clinical Trials): Merck, Boerhinger-Ingelheim, GlaxoSmithKline, Roche/Genentech, Bayer, Abbvie, EMD Serono, ALX Oncology, ISA Therapeutics, Ayala Therapeutics, Debiopharm; Honoraria: Merck, EMD Serono, BMS outside the submitted work. AS reports grants and personal fees from Novartis, Merck, BMS, JNJ, and Oncorus and grants from Symphogen, Roche, Northern Biologics, Regeneron, AstraZeneca MedImmune, ArrayBiopharma/Pfizer, GSK, Bayer, Surface Oncology, and Treadwell outside the submitted work. AH reports grants and other support from GSK, Merck, Eisai, and Novartis, as well as grants from MedImmune, Roche, Boehringer Ingelheim, Pfizer, Janssen, and BMS outside the submitted work. SMcD reports Co-founder/shareholder: AilseVax outside of submitted work. VC reports research grants: Astex

Pharmaceuticals, Cancer Research UK, NIHR and honoraria: Servier, outside of submitted work. ML reports educational grant from Pfizer and honoraria: Pfizer, EMD Serono, Roche, Carnall Farrar outside the submitted work. EE reports receiving research funding from Bristol Myers Squibb and Zymeworks; serving as a consultant or in an advisory role for Bristol Myers Squibb, Zymeworks, and Adaptimmune; and having an immediate family member employed by Merck, outside the submitted work. SVB is inventor on patents related to cell-free DNA mutation and methylation analysis technologies that have been licensed to Roche Molecular Diagnostics and Adela, respectively. SVB is a cofounder of, has ownership in, and serves in a leadership role at Adela. LLS reports personal fees from Merck, Pfizer, Celgene, AstraZeneca, Morphosys, Roche, Loxo, Oncorus, Symphogen, Seattle Genetics, GlaxoSmithKline, Voronoi, Treadwell Therapeutics, Arvinas, Tessa, Navire, Relay Therapeutics, Rubius, Janpix, Agios, and Treadwell Therapeutics and grants from Merck, Novartis, Bristol-Myers Squibb, Pfizer, Boerhinger-Ingelheim, GlaxoSmithKline, Roche/Genentech, Karyopharm, AstraZeneca, Astellas, Bayer, AbbVie, Amgen, Symphogen, Intensity Therapeutics, Mirati, Shattucks, and Avid outside the submitted work.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.04.014.

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