ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

ANNALS OF ONCOLOGY driving innovation in oncology

ORIGINAL ARTICLE

Tumor-agnostic plasma assay for circulating tumor DNA detects minimal residual disease and predicts outcome in locally advanced squamous cell carcinoma of the head and neck

N. Honoré[1,2], C. van Marcke[1,2], R. Galot[1,2], R. Helaers[3], J. Ambroise[4], A. van Maanen[5], A. Mendola[1], H. Dahou[1], E. Marbaix[6], P. Van Eeckhout[6], E. Longton[7], M. Magremanne[1,8], S. Schmitz[1,9], N. Limaye[10] & J.-P. Machiels[1,2]*

[1]Pôle oncologie, Institut de Recherche Clinique et Expérimentale, Université catholique de Louvain (UCLouvain), Brussels; [2]Department of Medical Oncology, Institut Roi Albert II, Cliniques universitaires Saint-Luc, Brussels; [3]Human Molecular Genetics, de Duve Institute, Université catholique de Louvain (UCLouvain), Brussels; [4]Center for Applied Molecular Technologies, Institute of Clinical and Experimental Research, Université catholique de Louvain (UCLouvain), Brussels; [5]Statistical Support Unit, Cliniques Universitaires Saint-Luc, Brussels; Departments of [6]Pathology; [7]Radiotherapy; [8]Maxillo-facial Surgery; [9]ENT and Head and Neck Surgery, Cliniques Universitaires Saint-Luc, Brussels; [10]Genetics of Autoimmune Diseases and Cancer, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium

Available online XXX

Background: Forty to fifty percent of patients with locally advanced squamous cell carcinoma of the head and neck (LA SCCHN) relapse despite multimodal treatment. Circulating tumor DNA (ctDNA) has the potential to detect minimal residual disease (MRD) after curative-intent therapy and to identify earlier which patients will progress. We developed a tumor-agnostic plasma ctDNA assay to detect MRD in unselected LA SCCHN with the aim of predicting progression-free survival (PFS) and overall survival without the need for tumor sequencing.

Patients and methods: A 26-gene next-generation sequencing panel was constructed that included the most frequently mutated genes in SCCHN and two HPV-16 genes. MRD was assessed in each patient through an in-house informatic workflow informed by somatic mutations identified in the corresponding pre-treatment plasma sample. The presence of MRD was defined as the detection of ctDNA in one plasma sample collected within 1-12 weeks of the end of curative treatment. The primary endpoint was the PFS rate at 2 years. At least 32 patients were planned for inclusion with the hypothesis that PFS at 2 years was >80% in MRD-negative patients and <30% in MRD-positive patients ($\alpha = 0.05$, $\beta = 0.9$).

Results: We sequenced DNA from 116 plasma samples derived from 53 LA SCCHN patients who underwent curative-intent treatment. ctDNA was detected in 41/53 (77%) patients in the pre-treatment samples. Out of these 41 patients, 17 (41%) were MRD positive after treatment. The 2-year PFS rate was 23.53% (9.9% to 55.4%) and 86.6% (73.4% to 100%) in MRD-positive and MRD-negative patients, respectively ($P < 0.05$). Median survival was 28.37 months (14.30 months-not estimable) for MRD-positive patients and was not reached for the MRD-negative cohort ($P = 0.011$).

Conclusions: Our ctDNA assay detects MRD in LA SCCHN and predicts disease progression and survival without the need for tumor sequencing, making this approach easily applicable in daily practice.

Key words: circulating tumor DNA (ctDNA), minimal residual disease (MRD), squamous cell carcinoma of the head and neck (SCCHN)

INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) is the seventh most common malignancy and affects around 600 000 patients per year worldwide.[1] Less than 60% of patients with locally advanced (LA) disease (Union for International Cancer Control stages III and IV) remain free of disease at 3 years despite aggressive multimodal local therapy with surgery and/or chemoradiation.[1-3]

Recent randomized trials that investigated treatment intensification in LA SCCHN failed to meet their primary endpoints.[2-4] These results may partly be explained by the absence of markers able to identify minimal residual disease (MRD) after curative-intent therapy, and such markers could potentially improve patient selection. Studies conducted in different cancer types have demonstrated that circulating tumor DNA (ctDNA) has the potential to identify patients

*Correspondence to: Prof. Jean-Pascal H. Machiels, Department of Medical Oncology, Institut Roi Albert II, Cliniques universitaires Saint-Luc and Institut de Recherche Clinique et Expérimentale, Université catholique de Louvain (UCLouvain), Avenue Hippocrate 10, 1200 Brussels, Belgium. Tel: +32-27645457

E-mail: jean-pascal.machiels@saintluc.uclouvain.be (J.-P. Machiels).

likely to recur with high specificity and sensitivity.[5-7] The early detection of patients at high or low risk of cancer relapse after curative-intent treatment could dramatically modify patient surveillance and therapeutic strategies.

SCCHN is heterogeneous with several disease locations. It has two main etiologies [tobacco/alcohol versus human papilloma virus (HPV)], and each one has a different prognosis. Genomic alterations are mainly found in tumor suppressor genes.[8,9] These factors make ctDNA detection challenging, and only limited data are available for SCCHN, particularly for HPV-negative SCCHN. Chera et al.,[10] using a multianalyte digital PCR assay, showed that HPV-16 ctDNA detection in plasma samples during post-treatment surveillance has a high positive predictive value (PPV) and a high negative predictive value (NPV) for diagnosing disease recurrence, with a median lead time of 3.9 months in patients with HPV-driven oropharyngeal cancer (OPC). Flach et al.[11] used a deep-sequencing personalized assay (RaDaR™) to detect ctDNA in post-surgery samples. In all patients who developed disease recurrence, ctDNA was detected before progression with lead times ranging from 3.6 to 8.4 months. To detect ctDNA, both approaches investigated personalized methodologies based on prior knowledge of the patient-specific tumor genomic landscape.

To the best of our knowledge, no studies have shown that a tumor-agnostic MRD assay can detect ctDNA with clinically meaningful specificity and sensitivity in unselected LA SCCHN. Such agnostic technologies have the advantage that they can detect ctDNA without the need for a tumor biopsy and do not require a costly and personalized assay for each patient. However, their sensitivity may be decreased given that several genes generally need to be sequenced, and deep sequencing does not have the same low limit of detection as PCR-based technologies.[12]

With the aim of developing an agnostic assay able to predict disease progression, we investigated an in-house 26-gene panel to diagnose MRD within 12 weeks of curative-intent therapy in unselected LA SCCHN. Our results demonstrate that early MRD detection is associated with shorter progression-free survival (PFS) and overall survival (OS).

PATIENTS AND METHODS

Patient cohort, study design, and inclusion criteria

After obtaining written informed consent, all SCCHN patients in our institution treated with standard-of-care protocols were prospectively enrolled into UCL-ONCO-2013 (2013/12FEV/047), a non-interventional biomarker trial collecting whole blood, plasma samples, and sometimes tumor biopsies, before, during, and after therapy (NCT02139020). The clinical characteristics and disease outcomes of each patient were also prospectively recorded in a secured database (REDCap[13]). The study was approved by our ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000).

Eligibility criteria were: patients (i) enrolled in UCL-ONCO-2013 between 2019 and 2022 with LA SCCHN [stages III/IVa/IVb p16-negative oropharyngeal, larynx, oral cavity, hypopharynx, and unknown primary cancers; and stage I (only N1)/II/III p16-positive OPC according to the eighth TNM (tumor—node—metastasis) classification], (ii) treated with curative-intent therapy according to standard of care, (iii) for whom pre- and post-treatment plasma and whole blood samples were available, and (iv) with no clinical disease progression from the initiation of curative-intent treatment until collection of the post-treatment plasma sample. The post-treatment plasma sample had to be collected within 12 weeks of the end of the curative treatment. Nasopharyngeal, salivary, and sinonasal cancers were excluded. HPV-driven OPCs were defined as tumors that were positive for HPV by *in situ* hybridization (ISH).

Study objective and endpoints

Our main objective was to develop a tumor-agnostic ctDNA assay to detect MRD within 12 weeks of the end of curative-intent treatment in unselected LA SCCHN. Our primary endpoint was the rate of PFS at 2 years in MRD-positive and MRD-negative patients who had ctDNA detected in their pre-treatment plasma sample. Secondary endpoints were PFS and OS in MRD-positive and MRD-negative patients.

MRD was defined as ctDNA detection in the post-treatment sample collected within 12 weeks of the end of the curative treatment. PFS was defined as the time interval between the date of the first day of curative treatment and the date of SCCHN progression, the date of last follow-up, or the date of death due to any cause. OS was defined as the time interval between the date of the first day of curative treatment until 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 curative-intent treatment and between 1 and 12 weeks after the end of curative-intent treatment. When possible, plasma samples were also collected during follow-up, every 2-3 months until relapse, or for up to 3 years following a patient's initial diagnosis. For each patient and time point, plasma was isolated from whole blood collected in two EDTA 7.5 ml tubes after double centrifugation (2000*g* 10 min and 2500*g* 15 min) within 30 min at 4°C. Plasma was stored immediately at −80°C until further use.

Cell-free DNA and germline DNA extraction

Cell-free DNA (cfDNA) was extracted from 4-6 ml of plasma using the Promega® Maxwell® RSC cfDNA LV Plasma Kit (RSC; Promega, Leiden, The Netherlands) and quantified with Qubit® (Thermo Fisher Scientific, Waltham, MA). Matched germline DNA was extracted for each patient with the Promega® Wizard® Genomic DNA Purification Kit (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). All sample characteristics and

quantification can be found in Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2023.09.3102.

cfDNA targeted next-generation sequencing and variant calling

Plasma samples were analyzed following our in-house workflow, as described in Figure 1. All pre-treatment plasma samples were first analyzed with Kraken2 (v. 2.1.2, database 'Standard', March 2023, Johns Hopkins University),[14] a taxonomic sequence classifier that assigns taxonomic labels to DNA sequences. A patient was considered HPV-16 positive if at least one read was assigned to the HPV-16 genome in the pre-treatment plasma sample, according to Kraken2. Otherwise, the patient was considered HPV-16 negative.

Pre- and post-treatment plasma samples of HPV-16 ctDNA-negative patients were analyzed using our gene panel consisting of the most frequently mutated genes in SCCHN according to literature[9,15-17] and our own in-house tumor sequencing data. Twenty-four genes plus two additional genes (*E6* and *E7*) from the HPV-16 genome were included in the panel (Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2023.09.3102). MRD was assessed in each patient through an in-house informatic workflow informed by somatic mutations identified in the corresponding pre-treatment plasma sample (see Supplementary Methods—Material S1, available at https://doi.org/10.1016/j.annonc.2023.09.3102). A variant was considered positive in the pre-treatment plasma sample if it was (i) supported by at least three reads, (ii) present in reads aligned in forward and reverse, (iii) passed modified Mutect2 quality filters, and (iv) present in $<1\%$ of healthy individuals in the public sequence databases (Gnomad). Read alignment for all variants was inspected manually. The list of all criteria can be found in Supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2023.09.3102, and only variants fulfilling all criteria were considered true somatic pre-treatment variants. A pre-treatment sample was considered ctDNA positive if at least one somatic variant was called. A variant was considered present in the post-treatment plasma sample if it was (i) previously identified in the pre-treatment sample, (ii) called in the Binary Alignment Map (BAM) file, and (iii) detected in three or more reads in the BAM files of the post-treatment plasma sample of the same patient. The ctDNA level was estimated to be 'high' if the higher allele frequency per sample was above the median of all variant allele frequencies (VAFs) per time point, and 'low' if below the median.

Digital droplet PCR (ddPCR) and HPV ISH are described in Supplementary Methods—Material S2, available at https://doi.org/10.1016/j.annonc.2023.09.3102.

Statistics

The primary endpoint was the PFS rate at 2 years in the population of patients who had ctDNA detected in the pre-treatment sample. The hypothesis was that the PFS rate at 2 years would be $<30\%$ for MRD-positive patients and $>80\%$ for MRD-negative patients. According to the two-sided Z-test (unpooled variance), a minimum of 32 pre-treatment ctDNA-positive patients with at least 2 years of follow-up were needed ($\alpha = 0.05$, power $= 0.9$).

PFS and OS were estimated using Kaplan–Meier methods. Patients without any event (progression or death) were censored at the date of last follow-up. The occurrence of another cancer was not considered to be an event. Treatment differences in PFS and OS were assessed using the log-rank test.

Cox proportional hazards regression models were used to assess the prognostic value of several parameters on OS and PFS. All analyses were done using SAS (v. 9.4, North Carolina State University) and R (v. 4.2.1, R Core team).

RESULTS

Patient characteristics

Between February 2019 and March 2022, 75 LA SCCHN patients were included in our prospective biomarker plasma

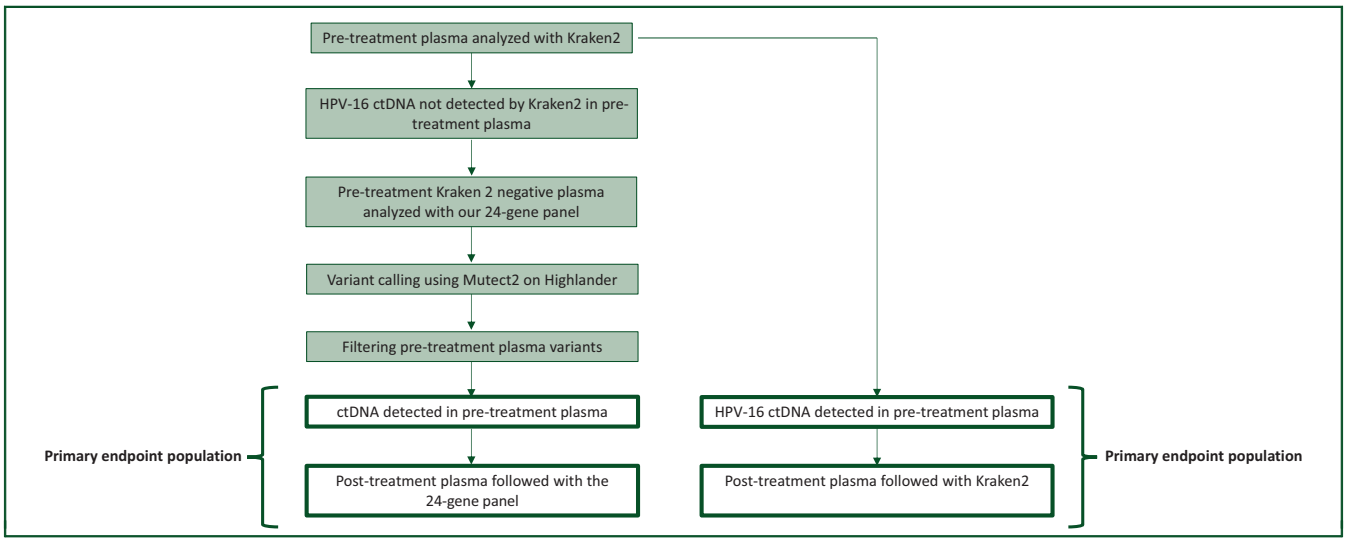

Figure 1. In-house bioinformatic workflow.
ctDNA, circulating tumor DNA; HPV, human papillomavirus.

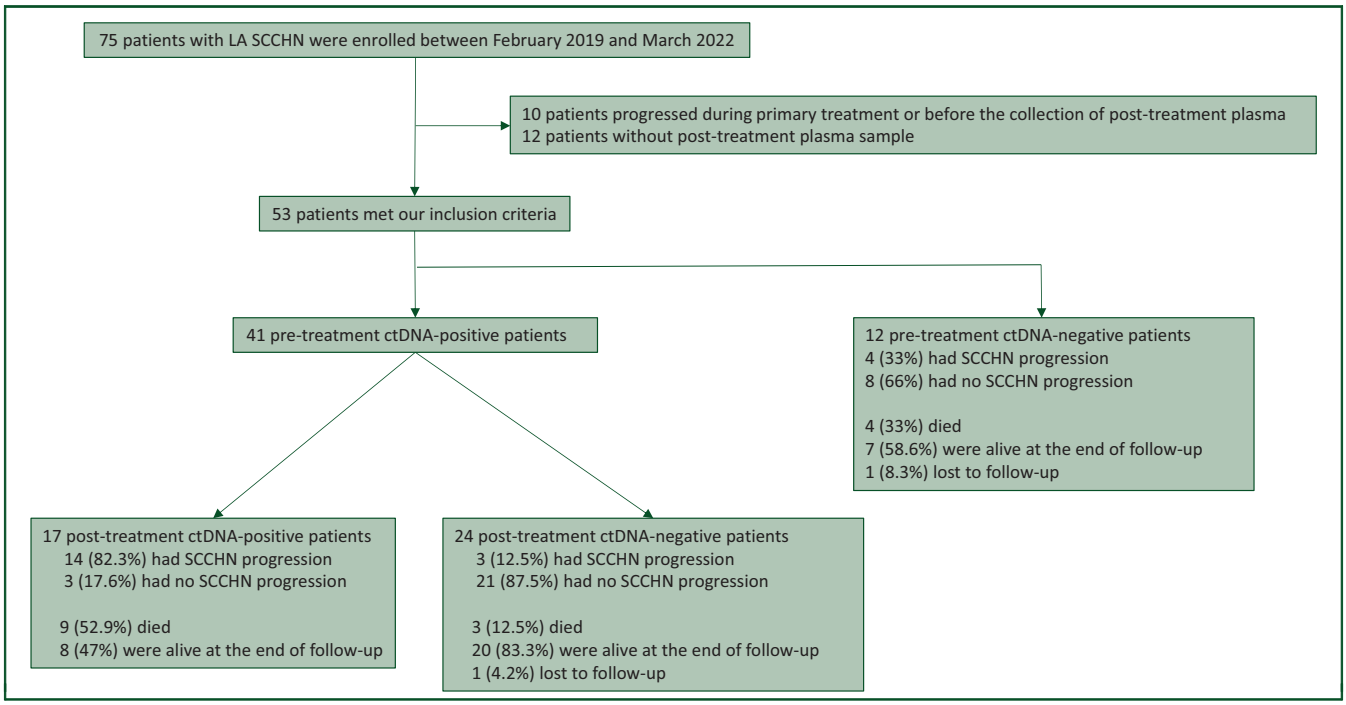

Figure 2. Patient workflow.
ctDNA, circulating tumor DNA; LA, locally advanced; SCCHN, squamous cell carcinoma of the head and neck.

collection trial. Fifty-three patients met our inclusion criteria (Figure 2) and had pre-treatment and post-treatment plasma samples available. Patient characteristics are described in Table 1 and Supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2023.09.3102. The most frequent tumor site was the oropharynx (n = 29, 54.7%). Seventeen (58.6%) patients had p16-positive OPC and, of these, nine were HPV positive by ISH. For one patient, ISH could not be carried out due to the insufficiency of tumor cells. Forty-nine (92.4%) and four (7.6%) patients were treated with curative-intent (chemo) radiation and surgery as primary treatment, respectively. The estimated median follow-up was 31.0 months (range 2.9-46.5 months). At 2 years, the PFS rate was 60.3% [95% confidence interval (CI) 48.0% to 75.8%] and OS was 78.0% (95% CI 67.2% to 90.4%). The median PFS was 34.7 months (1.7-44.0 months) and the median OS was not reached.

Post-treatment plasma samples were collected between weeks 1 and 12 (median 6 weeks) after the end of the curative-intent treatment.

Pre-treatment ctDNA detection rate

Overall, ctDNA was detected in 41 (77%) out of the 53 pre-treatment plasma samples (Figure 2).

The pre-treatment plasma samples of the 53 patients were first evaluated for the presence of HPV-16 ctDNA using Kraken2 (Figure 1). The nine patients with HPV-related OPC diagnosed by ISH, and the patient for whom ISH could not be carried out, were found to have circulating HPV-16 ctDNA in their pre-treatment samples. HPV ctDNA was not detected in the other patients.

Using the bioinformatic pipeline described in Figure 1, 31 patients (72.1%) out of the 43 HPV-16-negative patients by Kraken2 had at least one tumor variant in their pre-treatment plasma samples. Variants were found in the *TP53* (67.0%), *NOTCH1* (39.5%), *EGFR* (30.2%), *KMT2D* (25.5%), and *FAT1* (23.2%) genes. VAF ranged from 0.2% to 20.2% (median 0.87%) of circulating cfDNA. Details of the variants found in the pre-treatment samples are included in Supplementary Table S5, available at https://doi.org/10.1016/j.annonc.2023.09.3102. ctDNA positivity in pre-treatment plasma samples was not predictive of OS (P = 0.5) or progression (P = 0.66).

ctDNA detection within 12 weeks of completion of curative-intent treatment

Among the 41 pre-treatment ctDNA-positive patients, 17 (41.4%) had ctDNA detected in the paired post-treatment plasma sample collected within 12 weeks of the end of curative treatment. The VAFs ranged from 0.073% to 31.53% (median 0.29%) in post-treatment plasma samples. Of these 17 HPV-16-negative patients, 14 (82.3%) had disease progression [median time to progression after end of treatment 10 months (1-30 months)]. Twenty-four patients were post-treatment ctDNA negative, and 20 (83.3%) of these patients did not experience SCCHN relapse. The median time to disease progression for the four post-treatment ctDNA-negative patients who progressed was 14 months (4.8-27.4 months). The median lead time between ctDNA-positive plasma samples and the time of clinically or radiologically detected disease progression was 4.9 months (0.7-32.8 months). Details can be found in Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2023.09.3102.

Overall, for the 41 pre-treatment ctDNA-positive patients, our MRD ctDNA assay had a sensitivity of 77.8%,

Table 1. Patient characteristics

	Overall (n = 53)
Sex, *n* (%)	
Female	13 (24.5)
Male	40 (75.5)
Age (years)	
Median (min, max)	63.7 (41.1, 98.7)
Tobacco consumption (>10 pack years), *n* (%)	
No	14 (26.4)
Yes	39 (73.6)
Alcohol consumption (>2 units/day), *n* (%)	
No	22 (41.5)
Yes	31 (58.5)
Primary localization of tumor, *n* (%)	
Hypopharynx	8 (15.1)
Larynx	9 (17.0)
Oral cavity	5 (9.4)
Oropharynx	29 (54.7)
p16 negative	12 (41.3)
p16 positive	17(58.6)
HPV high risk ISH negative	7 (41.1)
HPV high risk ISH positive	9 (53)
HPV high risk ISH unknown	1 (5.8)
Unknown primary	2 (3.8)
Curative-intent treatment regimen, *n* (%)	
Primary chemoradiation	39 (73.6)
Primary radiation	10 (18.8)
Primary surgery	4 (7.6)
Staging according to eighth TNM edition, *n* (%)	
p-16-positive oropharynx cancer	17 (32.07)
I (T1-T2, N1)	9 (17.0)
II	3 (5.7)
III	5 (9.43)
L, OC, H, UP cancers, p-16-negative OPC	36 (67.92)
III	10 (18.86)
IVa	17 (32.1)
IVb	9 (17.0)
Performance status (ECOG), *n* (%)	
0	35 (66)
1	16 (30.2)
2	2 (3.8)
Disease progression, *n* (%)	
No	32 (60.4)
Yes	21 (39.6)

ECOG, Eastern Cooperative Oncology Group; H, hypopharynx cancer; HPV, human papillomavirus; ISH, *in situ* hybridization; L, larynx cancer; OC, oral cavity cancer; OPC, oropharyngeal cancer; TNM, tumor—node—metastasis; UP, unknown primary.

specificity of 86.9%, PPV of 82.3%, NPV of 83.3%, and accuracy of 79.1% for predicting disease progression. In the HPV-negative population with detectable ctDNA in their pre-treatment plasma samples (n = 31), ctDNA was detected in post-treatment plasma samples in 14 out of 17 patients who eventually relapsed and was not detected in 11 out of 14 patients who did not relapse. Therefore, in the HPV-negative population of this study, our assay showed a sensitivity of 82.4%, specificity of 78.6%, PPV of 82.3%, NPV of 78.6%, and accuracy of 80.7%. Details of our assay's performance in the different subgroups can be found in Supplementary Table S6, available at https://doi.org/10.1016/j.annonc.2023.09.3102.

Four patients were false negative in that no ctDNA was detected by our assay after treatment despite confirmed disease progression. One of these patients had an HPV OPC, and HPV-16 ctDNA was detected by ddPCR at 0.04 copies/ml in the Kraken2-negative post-treatment sample taken 1 week after treatment. One HPV-negative patient had no ctDNA detected in the post-treatment plasma sample taken at week 1. In this patient, ctDNA was subsequently detected in the post-treatment plasma sample taken 13 weeks after the first. Unfortunately, the other two patients refused further plasma collection. Three patients were false positive in that ctDNA was detected after treatment without SCCHN progression. We analyzed the sequential plasma samples and the evolution of VAFs when available. One patient had a variant detected 1 week after curative-intent chemoradiation. This patient also refused sequential plasma sampling, making it impossible to determine whether the variant disappeared thereafter. Interestingly, the other two patients developed non-SCCHN cancers during follow-up (Figure 3). The first developed a lung adenocarcinoma and the second a pancreatic carcinoma at 20 and 12 months of follow-up, respectively. These second cancers were not detectable on [^{18}F]2-fluoro-2-deoxy-D-glucose—positron emission tomography or on the computed tomography scans carried out at the time of SCCHN diagnosis. For both patients, we sequenced the SCCHN biopsies obtained at diagnosis. The two ctDNA variants found in the pre-treatment sample of the lung adenocarcinoma patient were not found in the SCCHN biopsy and remained present in the plasma after SCCHN treatment (Figure 3). They disappeared from plasma following surgery of the lung adenocarcinoma, suggesting that this was where the variants originated. In the patient with pancreatic adenocarcinoma, seven ctDNA variants were detected in the pre-treatment plasma sample. One of these variants was also found in the SCCHN biopsy but was cleared in the post-treatment plasma sample. However, the other six variants were not found in the SCCHN biopsy and were still present in the post-treatment plasma samples (Figure 3), suggesting that they originated from the pancreatic cancer.

The presence of MRD after curative treatment is associated with lower PFS and OS

For pre-treatment ctDNA-positive patients, the 2-year PFS rate was 23.5% (95% CI 10% to 55%) and 86.6% (95% CI 73.4% to 100%) in MRD-positive and MRD-negative patients, respectively ($P < 0.05$). Median PFS was 11 months for MRD-positive patients but was not reached for those who were MRD negative ($P < 0.0001$) (Figure 4A). Median survival was 28.4 months for MRD-positive patients but was also not reached for the MRD-negative group ($P = 0.011$) (Figure 4B).

We carried out sensitivity analyses for PFS and OS in three subgroups: the pre-treatment ctDNA-positive HPV-negative population (n = 31), the eligible HPV-negative population (n = 43), and the eligible population enrolled in this study (n = 53). For these two latter analyses, pre-treatment ctDNA-negative patients were included in the MRD-negative group. These additional analyses showed similar results to the ones observed in the primary endpoint population (Figure 4). For the pre-treatment ctDNA-positive HPV-negative population (n = 31), the 2-year PFS rate was

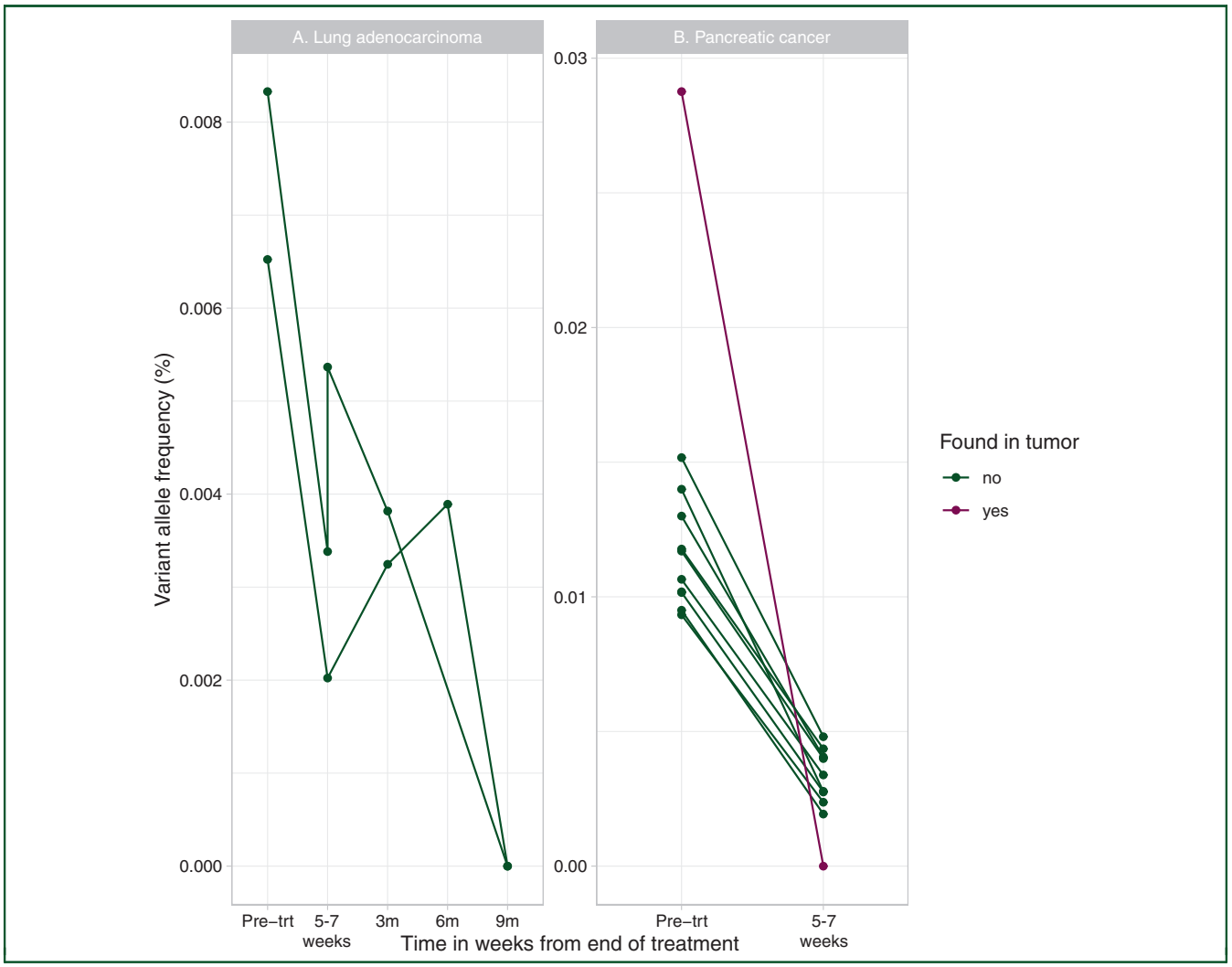

Figure 3. Kinetics of plasma variant detection in the two false-positive patients who developed another cancer. (A) Patient with lung adenocarcinoma. (B) Patient with pancreatic carcinoma. Variants found in the SCCHN are in green, and variants not found in the SCCHN are in red.
Pre-trt, pre-treatment; SCCHN, squamous cell carcinoma of the head and neck.

23.5% (95% CI 10.0% to 55.4%) and 85.7% (95% CI 69.2% to 100%) in the MRD-positive and -negative groups, respectively. Median PFS was 11.0 months (5.8-34.7 months) for HPV-negative MRD-positive patients but was not reached for those who were MRD negative ($P < 0.00033$) (Figure 4E). Median survival was 28.4 months (14.3 months-not estimable) for MRD-positive patients but was also not reached for the MRD-negative group ($P = 0.024$) (Figure 4F). For the eligible HPV-negative population ($n = 43$), regardless of their pre-treatment ctDNA status, PFS and OS remained worst in the MRD-positive group compared to the MRD-negative group ($P = 0.00026$ and $P = 0.068$, respectively) (Figure 4G and H).

On the 53 patients who met our clinical inclusion criteria, we carried out Cox univariate and multivariate analyses and included several prognostic factors: HPV status, disease stage, primary tumor location, Eastern Cooperative Oncology Group performance status, pre-treatment ctDNA level, type of primary curative-intent treatment, and the presence of MRD (Tables 2 and 3). In the univariate analysis, having a pre-treatment ctDNA level above the median value also significantly correlated with worst PFS (HR 4.3, 95% CI 1.82-10.39, $P < 0.001$) but not OS ($P = 0.153$). In the multivariate Cox model, MRD positivity remained an independent predictor of PFS (Table 3) (HR 12.2, 95% CI 2.59-57.50, $P = 0.002$) and OS (HR 12.22, 95% CI 1.58-94.6, $P = 0.017$) (Table 2).

DISCUSSION

We explored an in-house tumor-agnostic ctDNA assay to detect MRD after curative-intent treatment in unselected LA SCCHN. Our study met its primary endpoint having demonstrated a 2-year PFS of 23.5% (95% CI 10% to 55%) and 86.6% (95% CI 73.4% to 100%) in MRD-positive and MRD-negative patients, respectively ($P < 0.05$). With our approach, we were able to identify, with good confidence, a subgroup of patients with poor prognosis who may benefit from additional treatment and/or surveillance. Our results were confirmed in the HPV-negative subgroup, a patient population with less evidence, compared to the HPV-positive subtype.

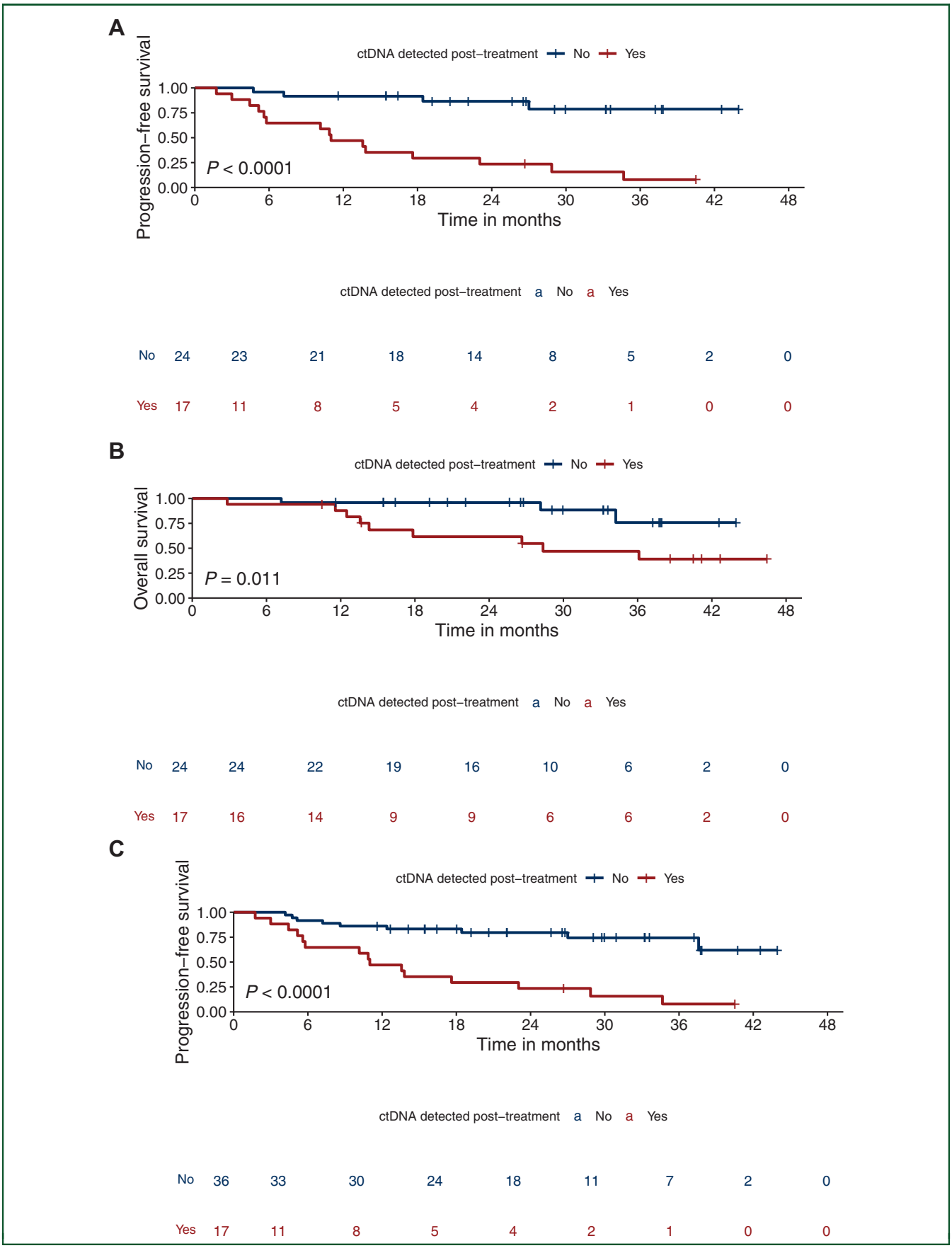

Figure 4. Overall survival and progression-free survival of MRD-positive and -negative patients. Kaplan—Meier estimate of progression-free survival (A, C, E, G) and overall survival (B, D, F, H) for MRD-positive and -negative patients in the pre-treatment ctDNA-positive population ($n = 41$) (A, B), in the entire population ($n = 53$) (C, D), in the HPV-negative pre-treatment ctDNA-positive population ($n = 31$) (E, F), and in the entire HPV-negative population ($n = 43$) (G, H).
ctDNA, circulating tumor DNA; MRD, minimal residual disease.

https://doi.org/10.1016/j.annonc.2023.09.3102

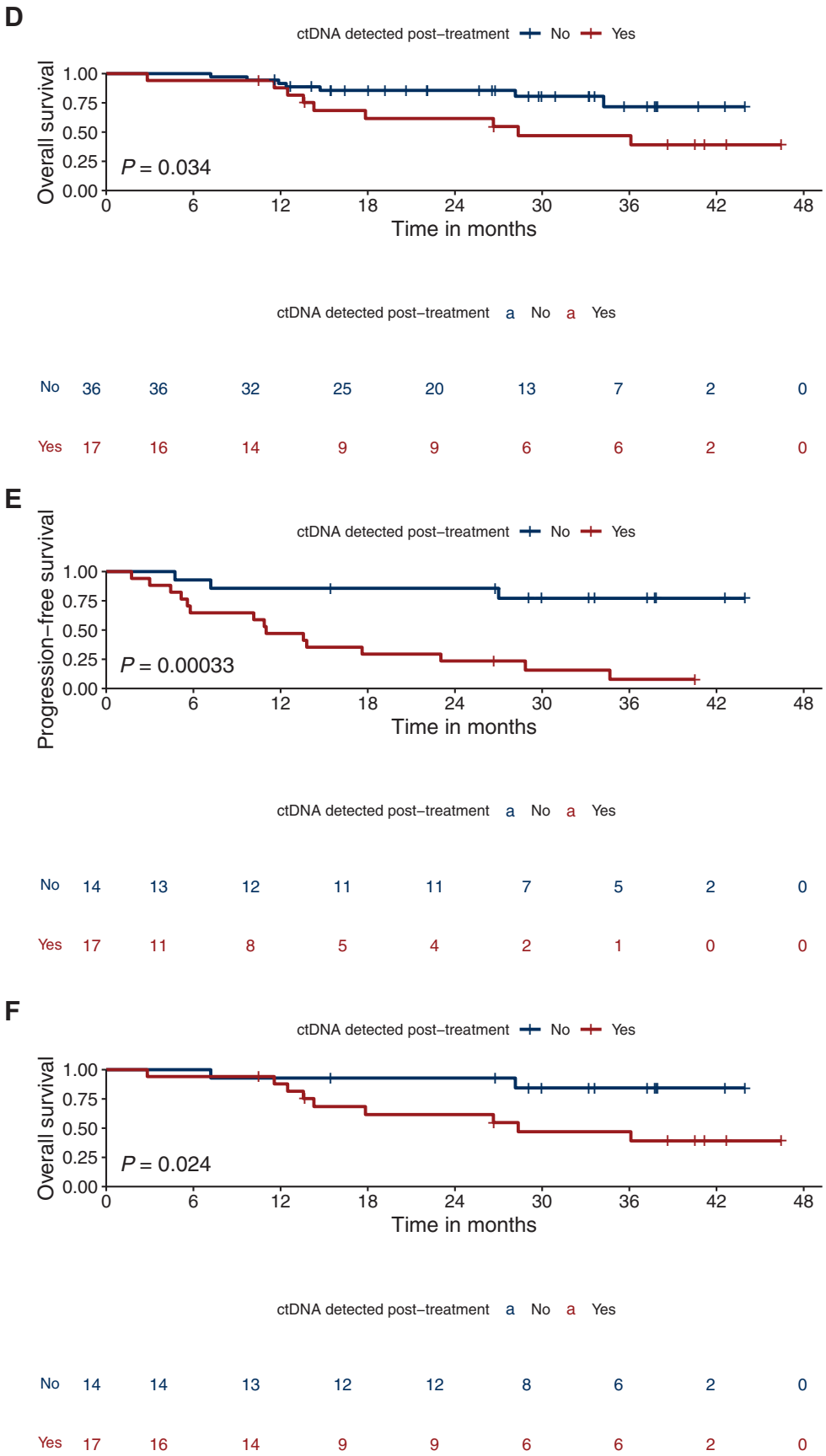

Figure 4. Continued.

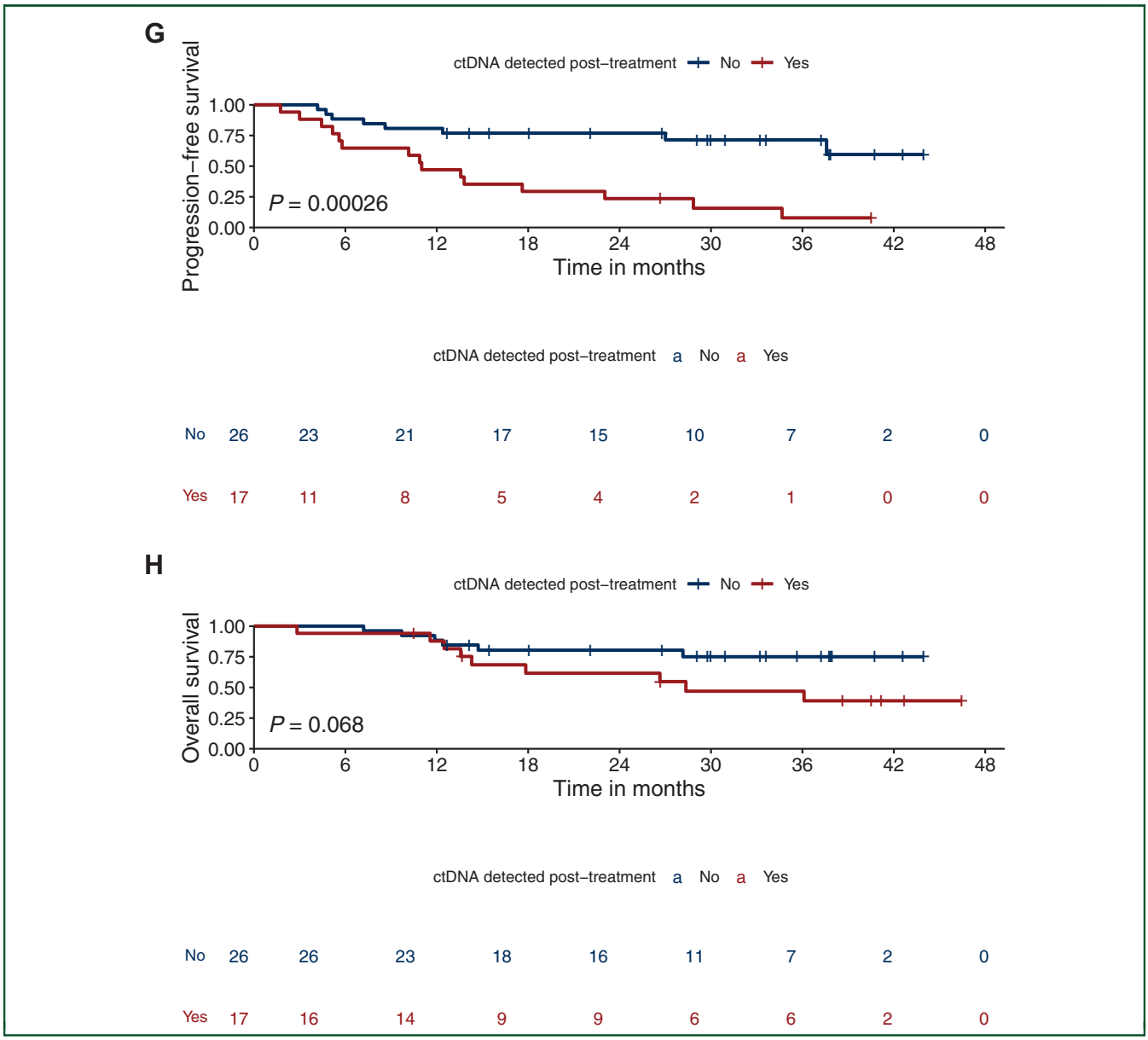

Figure 4. Continued.

ctDNA detection has shown promising results in other cancer types for treatment monitoring,[18] MRD detection,[19] and the identification of resistant clones.[20] However, most of these results were obtained with personalized tumor-informed assays which require a tumor biopsy for each patient and the development of a time-consuming and expensive patient-specific assay. Our methodology intends to loosen these constraints in the form of a single assay that can be applied to all LA HNSCC patients without the need for tumor sequencing.

Sanz Garcia et al. investigated MRD detection using another tumor-agnostic assay, Cancer Personalized Profiling by deep sequencing (CAPP-seq), in 29 patients with LA SCCHN.[21] Preliminary results were recently presented, but MRD was not shown to be associated with relapse-free survival within the year following treatment.

Since MRD was detected through an in-house bio-informatic workflow informed by the somatic mutations identified in the pre-treatment plasma samples, a possible downfall of our methodology is that pre-treatment ctDNA was only detected in 41 (77%) of the 53 included patients, with 100% ctDNA detection in HPV-driven OPC and 72% in HPV-negative SCCHN. However, personalized tumor assays are not without their own limitations. For example, they need a good-quality tumor biopsy for each patient in order to identify the variants of interest to be tracked in plasma, and this is only achieved in 70%[22,23] to 100%[11,24] of all SCCHN cases. This might be due to the low quality or quantity of tumor DNA extracted from the tumor biopsy or the inability to even obtain a tumor biopsy.[12]

Several factors may likely influence the pre-treatment sensitivity observed in our MRD ctDNA assay. One is the

Table 2. Identification of potential prognostic factors for overall survival

		Univariate			Multivariate		
Factor	Value	*P* value	HR	HR 95% CI	*P* value	HR	HR 95% CI
ECOG PS	0-1	0.044	2.85	1.028-7.880			
	2	0.995	0.00	0.00-NE			
Pre-treatment ctDNA quantity	High	0.073	3.37	0.0894-12.73	0.039	4.60	1.078-19.60
Primary location	Hypopharynx	0.665	1.43	0.286-7.131			
	Oral cavity	0.741	1.35	0.225-8.157			
	Oropharynx	0.692	0.76	0.188-3.027			
Staging TNM (eighth version)	IV	0.592	1.37	0.435-4.299			
Curative treatment	Chemoradiation	0.918	1.18	0.05-27.7			
	Radiotherapy	0.998	1	0.00-inf			
	Surgery	0.918	0.85	0.04-19.86			
Presence of ctDNA in the pre-treatment sample	Yes	0.439	0.63	0.200-2.011			
HPV status	Positive	0.152	0.33	0.075-1.499	0.218	3.76	0.457-30.92
Presence of MRD	Yes	0.153	2.10	0.758-5.817	0.017	12.22	1.578-94.62

Analysis carried out using Cox proportional hazards model. Higher bound for inclusion in multivariate is set to $P = 0.20$.
CI, confidence interval; ctDNA, circulating tumor DNA; ECOG PS, Eastern Cooperative Oncology Group performance status; HPV, human papilloma virus; HR, hazard ratio; MRD, minimal residual disease; NE, not evaluable; TNM, tumor—node—metastasis.

quantity of cfDNA obtained as a lower quantity will negatively impact the detection limit.[12,25] ctDNA also has a short half-life and is very labile,[26] and this may explain the variation in ctDNA quantity. ctDNA variants with a lower VAF could be missed as variants in genes not present in our panel. The minimal post-treatment VAF was 0.07%. This is comparable to other assays that are able to successfully detect MRD in other cancers.[27-29] As demonstrated in tumor-personalized assays,[11] pre-treatment tumor variants may be present at very low proportions. Flach et al.,[11] for example, reported that 20% of the samples presented with VAF <0.01%. This highlights a technical limitation of our study as next-generation sequencing is not, to this day, as sensitive as PCR-based techniques. Although this may explain the false-negative patients, it is interesting to note that our assay was still able to detect MRD with high sensitivity and specificity despite a higher limit of detection than with tumor-personalized approaches. A comparison of this non-personalized assay versus one that is personalized is certainly warranted in larger patient cohorts, together with an investigation of the clinical significance of tumor variants with VAF <0.01%.

Two false-positive patients developed second new cancers that were not detected radiologically at the time of SCCHN diagnosis. Our data indicate that these second cancers are most likely the reason behind the presence and persistence of post-treatment ctDNA diagnosed by our assay. This highlights the potential use of ctDNA as a cancer screening tool as is currently being investigated in large cohort studies.[30,31]

Our study was designed to investigate the prognostic impact of post-treatment ctDNA within 12 weeks of the end of curative treatment. The ability to detect patients with a high risk of relapse earlier may identify those who could benefit from adjuvant therapy or those who need close follow-up with salvage treatment in mind. However, the best time to assess MRD is unknown and varies from one study to another.[32] In SCCHN, the situation is even more complex given that the efficacy of primary chemoradiation is generally evaluated only 12 weeks later.[33] To our

Table 3. Identification of potential prognostic factors for progression-free survival

		Univariate			Multivariate		
Factor	Value	*P* value	HR	HR 95% CI	*P* value	HR	HR 95% CI
ECOG PS	0-1	0.029	2.57	1.104-5.968			
	2	0.992	0.00	0.00-NE			
Pre-treatment ctDNA quantity	High	0.090	2.28	0.472-6.615	0.030	3.12	1.113-8.734
Primary location	Hypopharynx	0.399	1.77	0.472-6.615			
	Oral cavity	0.352	2.04	0.454-9.201			
	Oropharynx	0.579	0.71	0.212-2.379			
Staging TNM (eighth version)	IV	0.710	1.19	0.483-2.913			
Curative treatment	Chemoradiation	0.764	0.66	0.04-10.42			
	Radiotherapy	0.368	7.09	0.10-503.66			
	Surgery	0.764	1.53	0.10-24.27			
Presence of ctDNA in the pre-treatment sample	Yes	0.653	1.28	0.434-3.791			
HPV status	Positive	0.019	0.18	0.041-0.752	0.671	1.48	0.242-9.060
Presence of MRD	Yes	<0.001	4.34	1.817-10.39	0.002	12.21	2.593-57.50

Analysis carried out using Cox proportional hazards model. Higher bound for inclusion in multivariate is set to $P = 0.20$.
CI, confidence interval; ctDNA, circulating tumor DNA; ECOG PS, Eastern Cooperative Oncology Group performance status; HPV, human papillomavirus; HR, hazard ratio; MRD, minimal residual disease; NE, not evaluable; TNM, tumor—node—metastasis.

knowledge, there are no studies that have evaluated the optimal timing of post-treatment ctDNA clearance after chemoradiation. Hilke et al.[34] detected MRD at 6-12 weeks after the end of chemoradiation in two out of eight patients who relapsed. Similarly, Burgener et al.[35] showed that the lack of ctDNA clearance 3 months after the end of curative therapy was strongly correlated with recurrence. In HPV OPC, Chera et al.[36] observed, despite rapid HPV clearance being a good prognostic factor, that patients with detectable HPV ctDNA at week 6 of chemoradiation did not have a poorer prognosis, and that for some patients it took >6 months to clear their HPV ctDNA. In a study investigating the kinetics of circulating Epstein—Barr virus (EBV) DNA by serial weekly plasma sampling in nasopharyngeal carcinoma, the authors noticed a rapid decline in plasma EBV DNA concentration after treatment; however, in some patients, an initial increase in the circulating EBV DNA level immediately after treatment initiation was observed, probably induced by the liberation of EBV DNA due to radiotherapy cell death.[37] In our study, post-treatment plasma samples were collected between weeks 1 and 12 (median 6 weeks) after the end of curative-intent treatment. Although we cannot exclude that the variability in timing may have influenced our results, we observed good correlation between the presence of MRD and disease progression. One false-positive patient had plasma collected 1 week post-chemoradiation, and it cannot be excluded that this patient was subsequently able to clear his ctDNA.

A limitation of this trial was the absence of serial sampling mainly because some of the patients refused further plasma collection. In the future, it would be interesting to evaluate the clearance of SCCHN ctDNA after chemoradiation on a weekly basis to determine optimal sampling timing. This is critical to understand both the biology and the clinical utility of ctDNA clearance after curative-intent therapy.

To our knowledge, Kraken2 has never been used in p16-positive SCCHN to detect HPV ctDNA. In clinical practice, HPV-driven tumors are detected through p16 immunochemistry with an estimated sensitivity of 96% and specificity of 92%.[38] In our cohort, 16 OPC patients had p16-positive staining and only 10 of these patients were HVP16 ctDNA positive according to Kraken2. We observed 100% concordance with ISH to determine HPV status. This highlights the value of our assay in determining which tumors are HPV-driven without the need for ISH. Kraken2 also enables the detection of HPV plasma DNA using the same DNA libraries and only one assay, thus avoiding the need for additional plasma and ctDNA.

One limitation of our trial is the low proportion of patients with HPV-driven tumors (19%). HPV-positive patients have a better prognosis compared to those with HPV-negative disease.[1] We observed one patient who was not diagnosed with MRD using Kraken2 at week 1 even though HPV ddPCR detected MRD at this time. However, in this patient, HPV-16 ddPCR detected only 0.04 copies/ml at this time point, which is the limit of detection (Supplementary Table S7, available at https://doi.org/10.1016/j.annonc.2023.09.3102). Since the number of HPV-positive patients in this trial was too low to assess the validity of our assay to detect MRD in HPV-positive tumors, larger cohorts are needed to fully assess the potential of this approach in this group of patients. It may also be the case that Kraken2 is not able to achieve the same sensitivity as HPV ddPCR.

In conclusion, our study demonstrates the feasibility of detecting MRD in LA SCCHN using a tumor-agnostic approach. Although we investigated this approach on a limited number of patients, the results are striking, particularly for HPV-negative SCCHN. Since this technology does not require tumor sequencing and is not personalized, it has the potential to be used widely and at a lower cost per patient. Our assay could stratify patients according to their risk of relapse and identify candidates for future interventional trials investigating escalation and de-escalation strategies.

ACKNOWLEDGEMENTS

The authors thank the members of the Genomics Platform at the University of Louvain for the use of the Highlander bioinformatic framework. We also thank the Fonds de la Recherche Scientifique—FNRS/Télévie, Keeping me Alive (ASBL), and Fondation Saint-Luc. We thank Thang, Salma, and Fadoua for their help. Finally, the authors thank Aileen Eiszele for writing and editing assistance, and the patients and their families who contributed to this work.

FUNDING

This work was supported by Belgian National Research Fund (FNRS)/Télévie, Keeping me Alive (ASBL), and Fondation Saint-Luc (Cliniques universitaires Saint-Luc, Brussels, Belgium) (no grant numbers).

DISCLOSURE

The authors have declared no conflicts of interest.

REFERENCES

1. Machiels JP, René Leemans C, Golusinski W, et al. Squamous cell carcinoma of the oral cavity, larynx, oropharynx and hypopharynx: EHNS-ESMO-ESTRO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2020;31(11):1462-1475.
2. Lee NY, Ferris RL, Psyrri A, et al. Avelumab plus standard-of-care chemoradiotherapy versus chemoradiotherapy alone in patients with locally advanced squamous cell carcinoma of the head and neck: a randomised, double-blind, placebo-controlled, multicentre, phase 3 trial. *Lancet Oncol*. 2021;22(4):450-462.
3. Burtness B, Haddad R, Dinis J, et al. Afatinib vs placebo as adjuvant therapy after chemoradiotherapy in squamous cell carcinoma of the head and neck: a randomized clinical trial. *JAMA Oncol*. 2019;5(8):1170-1180.
4. Machiels JP, Tao Y, Burtness B, et al. Primary results of the phase III KEYNOTE-412 study: pembrolizumab (pembro) with chemoradiation therapy (CRT) vs placebo plus CRT for locally advanced (LA) head and neck squamous cell carcinoma (HNSCC). *Ann Oncol*. 2022;33(suppl 7):S808-S869.
5. Tie J, Cohen JD, Wang Y, et al. Circulating tumor DNA analyses as markers of recurrence risk and benefit of adjuvant therapy for stage III colon cancer. *JAMA Oncol*. 2019;5:1710-1717.

6. Yang J, Gong Y, Lam VK, et al. Deep sequencing of circulating tumor DNA detects molecular residual disease and predicts recurrence in gastric cancer. *Cell Death Dis*. 2020;11(5):346.
7. Chaudhuri AA, Chabon JJ, Lovejoy AF, et al. Early detection of molecular residual disease in localized lung cancer by circulating tumor DNA profiling. *Cancer Discov.* 2017;7(12):1394-1403.
8. Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. *Nat Rev Cancer.* 2018;18(5):269-282.
9. Stransky N, Egloff AM, Tward AD, et al. The mutational landscape of head and neck squamous cell carcinoma. *Science*. 2011;333(6046):1157-1160.
10. Chera BS, Kumar S, Shen C, et al. Plasma circulating tumor HPV DNA for the surveillance of cancer recurrence in HPV-associated oropharyngeal cancer. *J Clin Oncol*. 2020;38(10):1050-1058.
11. Flach S, Howarth K, Hackinger S, et al. Liquid Biopsy For Minimal Residual Disease Detection In Head And Neck Squamous Cell Carcinoma (LIONESS)-a personalised circulating tumour DNA analysis in head and neck squamous cell carcinoma. *Br J Cancer.* 2022;126(8):1186-1195.
12. Galot R, Machiels JH. Current applications and challenges of circulating tumor DNA (ctDNA) in squamous cell carcinoma of the head and neck (SCCHN). *Cancer Treat Rev.* 2020;85:101992.
13. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (Redcap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform*. 2009;42(2):377-381.
14. Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. *Genome Biol*. 2019;20(1):257.
15. Agrawal N, Frederick MJ, Pickering CR, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science*. 2011;333(6046):1154-1157.
16. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer.* 2011;11(1):9-22.
17. Pickering CR, Zhang J, Yoo SY, et al. Integrative genomic characterization of oral squamous cell carcinoma identifies frequent somatic drivers. *Cancer Discov.* 2013;3(7):770-781.
18. Remon J, Besse B, Aix SP, et al. Osimertinib treatment based on plasma T790M monitoring in patients with EGFR-mutant non-small-cell lung cancer (NSCLC): EORTC Lung Cancer Group 1613 APPLE phase II randomized clinical trial. *Ann Oncol*. 2023;34(5):468-476.
19. Jackson-Spence F, Toms C, O'Mahony LF, et al. Imvigor011: a study of adjuvant atezolizumab in patients with high-risk MIBC who are ctDNA+ post-surgery. *Future Oncol*. 2023;19(7):509-515.
20. Huang CT, Lin CA, Su TJ, et al. Monitoring of T790M in plasma ctDNA of advanced EGFR-mutant NSCLC patients on first- or second-generation tyrosine kinase inhibitors. *BMC Cancer.* 2023;23(1):234.
21. Sanz Garcia E, Zou J, Avery L, et al. Multimodal detection in plasma of molecular residual disease (MRD) in locally advanced head and neck squamous cell carcinoma (LA-HNSCC). *J Clin Oncol*. 2023;41(suppl 16):6065.
22. Kogo R, Manako T, Iwaya T, et al. Individualized circulating tumor DNA monitoring in head and neck squamous cell carcinoma. *Cancer Med*. 2022;11(21):3960-3968.
23. Economopoulou P, Spathis A, Kotsantis I, et al. Next-generation sequencing (NGS) profiling of matched tumor and circulating tumor DNA (ctDNA) in head and neck squamous cell carcinoma (HNSCC). *Oral Oncol*. 2023;139:106358.
24. Wang Y, Springer S, Mulvey CL, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Sci Transl Med*. 2015;7(293):293ra104.
25. Diefenbach RJ, Lee JH, Menzies AM, et al. Design and testing of a custom melanoma next generation sequencing panel for analysis of circulating tumor DNA. *Cancers (Basel)*. 2020;12(8):2228.
26. Papadopoulos N. Pathophysiology Of ctDNA release into the circulation and its characteristics: what is important for clinical applications. *Recent Results Cancer Res*. 2020;215:163-180.
27. Gale D, Heider K, Ruiz-Valdepenas A, et al. Residual ctDNA after treatment predicts early relapse in patients with early-stage non-small cell lung cancer. *Ann Oncol*. 2022;33(5):500-510.
28. Azad TD, Chaudhuri AA, Fang P, et al. Circulating tumor DNA analysis for detection of minimal residual disease after chemoradiotherapy for localized esophageal cancer. *Gastroenterology.* 2020;158(3):494-505.E496.
29. Mcduff SGR, Hardiman KM, Ulintz PJ, et al. Circulating tumor DNA predicts pathologic and clinical outcomes following neoadjuvant chemoradiation and surgery for patients with locally advanced rectal cancer. *JCO Precis Oncol*. 2021;(5):123-132.
30. Cao M, Shi J, Xia C, et al. Efficacy Of ctDNA methylation combined with traditional detection modality to detect liver cancer among high-risk patients: a multicenter diagnostic trial. *Chin J Cancer Res*. 2023;35(1):58-65.
31. Lenaerts L, Che H, Brison N, et al. Breast cancer detection and treatment monitoring using a noninvasive prenatal testing platform: utility in pregnant and nonpregnant populations. *Clin Chem*. 2020;66(11):1414-1423.
32. Kasi PM, Fehringer G, Taniguchi H, et al. Impact Of circulating tumor DNA-based detection of molecular residual disease on the conduct and design of clinical trials for solid tumors. *JCO Precis Oncol*. 2022;6:E2100181.
33. Mehanna H, Wong WL, Mcconkey CC, et al. PET-CT surveillance versus neck dissection in advanced head and neck cancer. *N Engl J Med*. 2016;374(15):1444-1454.
34. Hilke FJ, Muyas F, Admard J, et al. Dynamics of cell-free tumour DNA correlate with treatment response of head and neck cancer patients receiving radiochemotherapy. *Radiother Oncol*. 2020;151:182-189.
35. Burgener JM, Zou J, Zhao Z, et al. Tumor-naïve multimodal profiling of circulating tumor DNA in head and neck squamous cell carcinoma. *Clin Cancer Res*. 2021;27(15):4230-4244.
36. Chera BS, Kumar S, Beaty BT, et al. Rapid clearance profile of plasma circulating tumor HPV type 16 DNA during chemoradiotherapy correlates with disease control in HPV-associated oropharyngeal cancer. *Clin Cancer Res*. 2019;25(15):4682-4690.
37. Lo YM, Leung SF, Chan LY, et al. Kinetics of plasma Epstein-Barr virus DNA during radiation therapy for nasopharyngeal carcinoma. *Cancer Res*. 2000;60(9):2351-2355.
38. Simoens C, Gheit T, Ridder R, et al. Accuracy of high-risk HPV DNA PCR, P16((INK4a)) immunohistochemistry or the combination of both to diagnose HPV-driven oropharyngeal cancer. *BMC Infect Dis*. 2022;22(1):676.