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Circulating Cell-Free DNA Concentration as a Biomarker in Head and Neck Cancer

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ABSTRACT

Objective: Head and neck squamous cell carcinoma (HNSCC), particularly human papillomavirus (HPV) -negative HNSCC, poses a significant clinical challenge due to late diagnoses and poor survival. This study evaluates the potential of circulating cell-free DNA (ccfDNA) as a minimally invasive biomarker for diagnosis, prognosis and disease monitoring in HNSCC.

Methods: We conducted a multicentre, prospective study enrolling 85 patients across all disease stages and 28 healthy controls, using two quantification ccfDNA methods: fluorometry (Qubit) and quantitative real-time polymerase chain reaction (qPCR).

Results: Baseline plasma ccfDNA concentrations were significantly elevated in HNSCC patients compared to healthy controls, with an area under the curve of 0.705. Higher ccfDNA levels were observed in early-stage HNSCC patients. While ccfDNA levels correlated with age, no significant associations were found with tumour stage or location. Patients with lower post-treatment ccfDNA levels demonstrated longer median progression-free survival (PFS) (16.37 months vs. 9.63 months, $p < 0.05$). Longitudinal analysis of locally advanced HNSCC revealed significant inter-patient variability in ccfDNA kinetics.

Conclusions: Our study demonstrates the potential value of fluorometric ccfDNA quantification as a diagnostic, prognostic and monitoring biomarker for HNSCC. However, further well-designed studies must be carried out to enhance the clinical utility of ccfDNA as a biomarker for HNSCC management.

1 | Introduction

Head and neck squamous cell carcinoma (HNSCC) represents a significant global concern, constituting 3.95% of all cancer-related deaths in 2022 (Bray et al. 2024) with over 770,000 new cases diagnosed annually worldwide. Historically, the typical profile of these patients included predominantly elderly men in their seventh decade of life, with a history of heavy tobacco and alcohol abuse (Beynon et al. 2018). Recently, new patterns of the disease have begun to emerge, broadening the spectrum of affected patient populations, including non-smokers and non-drinkers (Gillison et al. 2015; Mehanna et al. 2013). However, the predominant and most common type remains HPV-negative HNSCC, strongly associated with the aforementioned traditional risk factors. Despite current advances in multimodal management, this subgroup continues to be characterised by poor early diagnosis and survival rates (Hashim et al. 2019), with limited improvements in 5-year survival rates over the past few decades (Gatta et al. 2015; León et al. 2021; Sharkey Ochoa et al. 2022). To address this, efforts are underway to enhance current approaches with the introduction of precision oncology and individualized treatment in routine clinical practice.

In this context, liquid biopsy has positioned itself as a novel tool to trace and understand the alterations that occur during tumorigenesis, providing clinicians with an attractive and minimally invasive strategy for cancer management. Thus, several molecular components can be studied through it, providing a wide variety of information related to the genes, molecular alterations and pathways involved in cancer development, reflecting the overall state of the tumour and allowing for real-time monitoring, something that cannot be achieved with the solid tissue biopsy (Honoré et al. 2023; Sanz-Garcia et al. 2024).

Between the wide variety of liquid biopsy biomarkers in blood, circulating cell-free DNA (ccfDNA) represents one of the most investigated diagnostic, prognostic and therapeutic marker in cancer (Christensen et al. 2019; Zviran et al. 2020). It mainly consists of double-stranded DNA molecules with an average length of approximately 165 base pairs (bp), or multiples thereof and can be released into the bloodstream by normal and/or tumoral cells through mechanisms such as apoptosis, necrosis, or active release (Elazezy and Joosse 2018; Jahr et al. 2001). Since high levels of ccfDNA have been observed in cancer patients (Leon et al. 1977; Mattox et al. 2023), numerous studies have suggested the clinical value of ccfDNA quantification as a potential tumour biomarker (Dziadziuszko et al. 2022; Tissot et al. 2015). However, in head and neck cancer, the evidence remains scarce (Desai et al. 2018; Shukla et al. 2013).

The aim of this study was to assess the potential clinical utility of ccfDNA concentration as a biomarker for the diagnosis, prognosis and monitoring of HNSCC.

2 | Material and Methods

2.1 | Study Design and Participants

We conducted a multicentre, prospective case–control study including newly diagnosed (treatment-naïve) and recurrent HPV-negative HNSCC patients diagnosed between March 2021 and August 2024 at the Departments of Medical Oncology at Complexo Hospitalario Universitario de Santiago de Compostela (CHUS), Radio-Oncology at Hospital Lucus Augusti of Lugo (HULA), Oral and Maxillofacial Surgery at Complexo Hospitalario Universitario of A Coruña (CHUAC) and Radio-Oncology at Centro Oncológico de Galicia (COGA). Patients younger than 18 years, those with autoimmune diseases, history of cancer within the last 5 years or another tumour at baseline diagnosis were excluded. All tumours were staged according to the 8th tumour, node and metastasis (TNM) classification system (Amin et al. 2017). All participants provided written informed consent for a protocol approved by the Research Ethics Committee Networks in Galicia (Ref. No. 2018/003) before enrolling in the study. The approved protocol adhered to the principles of the Declaration of Helsinki.

2.2 | Sample Collection and Processing

Peripheral whole blood samples were collected and processed within 24–72 h, from eighty-five HNSCC patients and from 28 healthy individuals into Streck Cell-free DNA BCT tubes (Streck Corporate, La Vista, NE, USA). In addition to baseline sample collection, blood samples were also collected at different timepoints from patients with locally advanced (LA)-HNSCC. Sampling timepoints in LA-HNSCC patients were as follows: baseline pre-treatment (S1), 4–6 weeks post-surgery (if applicable) (S2), 2–3 weeks post-treatment follow-up (S3), 10 weeks post-treatment follow-up (S4) and progression of the disease (PD) (Figure 1). From all blood samples, plasma was obtained using a two-step centrifugation process at room temperature. Initially, centrifugation was performed at 1600 *g* for 10 min to isolate the plasma from the rest of the blood. Subsequently, the isolated plasma was subjected to a second centrifugation step at 5500 *g* for 10 min to eliminate the cellular debris. Immediately following centrifugation, plasma samples were divided into 5 mL aliquots and stored at −80°C until ccfDNA isolation. All samples were processed following the same protocol. Throughout the study, a total of 192 patients' blood samples were collected.

2.3 | Plasma ccfDNA Isolation and Quantification

Total ccfDNA was extracted from 3 to 6 mL of plasma using the QIAamp DNA Mini Blood Kit (Qiagen, Hilden, Germany) and QIAGEN Vacuum Manifold QIAvac 24 Plus pump, according to manufacturer instructions (Meddeb et al. 2019). Following extraction, the ccfDNA was eluted in LoBind tubes (Eppendorf

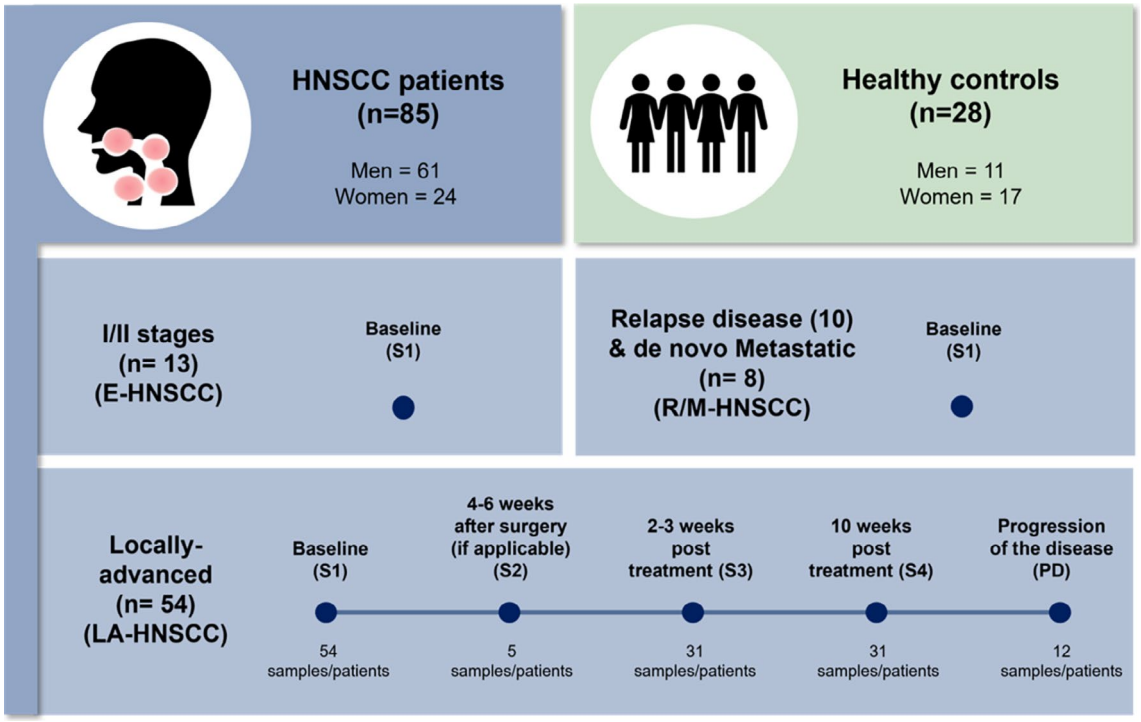


FIGURE 1 | Schematic representation of the different subgroups of HNSCC patients and the timepoints in which follow-up samples were collected for the LA-HNSCC group. E-HNSCC, early head and neck squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; LA-HNSCC, locally advanced head and neck squamous cell carcinoma; R/M-HNSCC, relapse/de novo metastatic head and neck squamous cell carcinoma.

AG, Hamburg, Germany) with 20–75 μL of nuclease-free water and then stored at −20°C for preservation.

CcfDNA concentrations were determined using two quantification methods. The primary method utilized the Qubit Fluorometer 4.0 (Thermo Fisher Scientific, Waltham, MA) in combination with the Qubit 1X dsDNA High Sensitivity (HS) Assay Kit (Invitrogen, Life Technologies, Carlsbad, CA). Additionally, as a secondary objective, quantitative real-time polymerase chain reaction (qPCR) was employed to compare measurements for a subset of baseline samples from the LA-HNSCC cohort ($n = 53$) and the cohort of controls. This qPCR analysis targeted the human telomerase reverse transcriptase (*hTERT*) single-copy gene (Thermo Fisher Scientific, Waltham, MA, USA) and served to evaluate the correlation between the two quantification methods.

For the first quantification method, 1 μL of each sample was diluted into 199 μL of a Qubit working solution prior to measurement in the Qubit Fluorometer 4.0, following the manufacturer's instructions. For the second quantification approach, plasma ccfDNA concentration was determined by using a previously reported real-time quantitative assay targeting the *hTERT* gene (Mondelo-Macía et al. 2021). The results obtained in nanograms per millilitre (ng/mL) using both techniques were standardis-Currened to genome equivalents (GE/mL).

Statistical analyses and data visualisations were performed using RStudio version 4.1.2 (2021-11-01) and GraphPad Prism 8.4.2. Descriptive statistics were reported as medians with interquartile ranges (IQR; first to third quartile, Q1–Q3) for continuous variables, and as absolute frequencies (n) and percentages (%) for categorical variables. Comparisons of ccfDNA concentrations between groups were conducted using the Mann–Whitney *U* test for binary variables and the Kruskal–Wallis test for variables with more than two categories. Diagnostic performance of ccfDNA as a biomarker was assessed using receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUROC) was used to assess discrimination ability, while the cut-off value for ccfDNA concentration was selected based on visual inspection of the ROC curve data to achieve an optimal balance between sensitivity and specificity. Spearman's rank correlation test was applied to evaluate the correlation between ccfDNA concentration and age. For survival analysis on the LA-HNSCC cohort, the Kaplan–Meier method was applied to evaluate progression-free survival (PFS) and overall survival (OS), with patients dichotomised into groups based on the median ccfDNA concentration. Spearman test and Bland–Altman analysis was used to evaluate a pairwise correlation between the different strategies to quantify the ccfDNA, by fluorometry and qPCR. All tests were two-tailed and a p value of < 0.05 was considered statistically significant.

2.4 | Treatment Efficiency Evaluation

For the LA-HNSCC cohort, treatment efficacy was evaluated at the S4 timepoint using the RECIST 1.1 criteria (Response Evaluation Criteria in Solid Tumours) (Eisenhauer et al. 2009), which classify tumours as complete response (CR), partial response (PR), progressive disease (PD), or stable disease (SD). The response was assessed using the imaging test performed closest to the S4 timepoint, ensuring an accurate reflection of treatment impact at that stage.

TABLE 1 | Baseline characteristics of included patients and controls.

Variables	HNSCC ($n=85$)		Controls ($n=28$)	
Age (years)				
Median (±SD)	65 (9.38)		64 (2.92)	
Range	45–90		47–74	
	n	**%**	***n***	**%**
< 65	42	49.41	16	57.14
> 65	43	50.59	12	42.86
Gender				
Male	61	71.76	11	39.29
Female	24	28.24	17	60.71
Ethnicity				
Caucasian	85	100.00	28	100.00
Smoking history				
Never	18	21.18	16	57.14
Former	24	28.24	7	25.00
Light	2	2.35	2	7.14
Moderate	17	20.00	3	10.71
Heavy	24	28.24		
Alcohol consumption				
Never	38	44.71	14	50.00
Former	7	8.23		
Current	38	44.71	14	50.00
NA	2	2.35		
Stage				
Early (I–II)	13	15.29		
Locally advanced (III–IVa/b)	54	63.53		
Relapse (locoregional/ distant metastasis)	10	11.76		
Metastatic (de novo)	8	9.41		
Location of primary tumour				
Oral cavity	38	44.71		
Oropharynx	20	23.53		
Hypopharynx	12	14.12		
Larynx	12	14.12		
No specific	3	3.53		
Histological grade				
Well	11	12.94		
Moderate	48	56.47		
Poor	14	16.47		
NA	12	14.12		

Abbreviations: NA, not available; SD, standard deviation.

2.5 | Survival Analysis

In the LA and R/M-HNSCC cohort, PFS was defined as the interval from diagnosis to disease progression or death from any cause. For patients who had not experienced disease progression and were alive at the end of the follow-up period, the date of their most recent health record review was used as the censoring point. OS was defined as the time from diagnosis to death from any cause, with data censored at the date of the last follow-up visit for patients who were still alive at the end of the follow-up period.

3 | Results

3.1 | Patient Characteristics

A total of 85 HNSCC patients and 28 healthy controls were enrolled in this study. The demographic and histopathological characteristics of the patients and controls are outlined in Table 1. The median age was 65 years (range: 45–90 years), with the majority of cases being males (71.76%) compared to females (28.24%), all suffering from squamous cell carcinoma as the histological type of head and neck cancer. Among the subjects, 78.82% had a history of smoking (current or former), and 44.71% reported active alcohol consumption. Regarding the stages of the disease at the time of diagnosis, the patients were classified into four cohorts: early-stage (I–II) HNSCC (E-HNSCC, $n=13$), locally advanced (stage III–IVa/b) (LA-HNSCC, $n=54$); relapse disease (locoregional recurrence and/or distant metastasis) (R-HNSCC, $n=10$) and de novo metastatic patients (M-HNSCC, $n=8$).

For the R/M-HNSCC group, who received the same palliative treatment regimen, 83.3% (15/18) of the patients experienced progressive disease, and 55.6% (10/18) died. However, after accounting for one death unrelated to cancer, the median OS for this group was 14.57 months. Regarding survival analysis, the median PFS for the R/M-HNSCC group was 6.62 months, with a range from 2.14 to 25.53 months. In the LA-HNSCC group, 27.78% (15/54) of the patients experienced PD, and 14 patients died (16.47%). The median OS was 17.59 months, and the median PFS was 15.88 months (range: 1.08–39.91 months).

3.2 | Plasma ccfDNA as a Potential Diagnostic Biomarker for HNSCC

The mean (±SD) concentration at diagnosis (S1) for the combined cohort of all HNSCC cases ($n=85$) was 2147 ± 1617 GE/mL, ranging from 389.1 to 9576 GE/mL, with a median

concentration of 1684 GE/mL (95% CI, 1347–1926). These values were significantly higher ($p < 0.0010$) compared to the control group, which had a mean concentration of 1235 ± 345.1 GE/mL, a median of 1233 GE/mL (95% CI, 1029–1376), and a range of 695.5 to 2227 GE/mL (Figure 2A). Analysing the concentration values for the different subgroups of HNSCC patients, the mean concentration for E-HNSCC patients was 2415 ± 1232 GE/mL, ranging from 1052 to 5657 GE/mL, with a median concentration of 2232 GE/mL (95% CI, 1394–3111), showing a significant difference in comparison with controls ($p < 0.0001$). For LA-HNSCC ($n = 54$), the mean concentration was 2105 ± 1661 GE/mL, ranging from 389.1 to 9576 GE/mL and a median of 1641 (95% CI, 1323–2020). For the R and M-HNSCC groups, the mean ccfDNA levels were 1592 ± 1169 and 2688 ± 2279 GE/mL, respectively, with ranges of 588.6 to 4727 for R-HNSCC and 737.6 to 7205 for M-HNSCC. The median ccfDNA level was 1224 GE/mL (95% CI, 945.5–1818) for R-HNSCC patients ($n = 10$) and 1682 GE/mL (95% CI, 737.6–7205) for M-HNSCC patients ($n = 8$). In both cases, although this data was higher than in controls, the differences were not statistically significant (Figure 2B).

In addition, ccfDNA levels were evaluated according to different patient and clinicopathological characteristics. Regarding age (< 65 years vs. ≥ 65 years), a statistically significant difference in ccfDNA concentrations was observed between these groups ($p = 0.024$) (Figure S1). Subsequently, subgroup analysis was performed for patients with naïve diagnosed HNSCC ($n = 75$) according to the following clinicopathological characteristics: primary tumour anatomic location (oral cavity vs. oropharynx vs. hypopharynx, vs. and larynx), tumour size (T1–T2 vs. T3–T4), N stage (N0 vs. N+), distant metastasis (M0 vs. M1) and grade of differentiation (well vs. moderately vs. poorly differentiated) (Figures S2–S6). A statistically significant difference was observed between the N+ and N0 subgroups with respect to ccfDNA concentrations ($p = 0.0202$).

3.3 | Diagnostic Value of Plasma ccfDNA

To calculate the diagnostic accuracy of ccfDNA levels, we performed a ROC analysis comparing the baseline samples of the HNSCC patients with the controls as shown in Figure 3A. This analysis yielded an AUC of 0.705 (95% CI: 0.610–0.799, $p = 0.0012$). Establishing the cut-off at 1451 GE/mL resulted in an overall sensitivity of 60% and a specificity of 82.14% for cancer diagnosis. For the E-HNSCC group, using a cut-off of 1385 GE/mL, the AUC was 0.863 (95% CI: 0.725–0.999, $p = 0.0002$), with a sensitivity of 84.62% and a specificity of 71.43% (Figure 3B). Lastly, the ROC curve for advanced HNSCC patients (stage III–IV) using a cut-off of 1451 GE/mL yielded an AUC of 0.671 (95 IC: 0.565–0.777, $p = 0.0088$), with a sensitivity of 56.72% and a specificity of 82.14% (Figure 3C).

3.4 | Prognostic Value of Plasma ccfDNA Levels in Prospective LA-HNSCC Cohort

We explored the prognostic value of ccfDNA levels in our cohort of patients with LA-HNSCC at three time points (S1, S3 and S4) by dichotomizing the cohort into 'High' and 'Low' ccfDNA groups, using the median of ccfDNA at each sample collection as threshold: 1641 GE/mL (S1), 1758 GE/mL (S2) and 1638 GE/mL (S3) (Table S1). Significant differences were observed at S4 for PFS, while no significant differences were found at S1 and S3 time points (Figure 4).

3.5 | Longitudinal Monitoring of ccfDNA Levels and the Response to Therapy in LA-HNSCC

We examined the value of ccfDNA kinetics in the group of LA-HNSCC as a prognostic biomarker during treatment

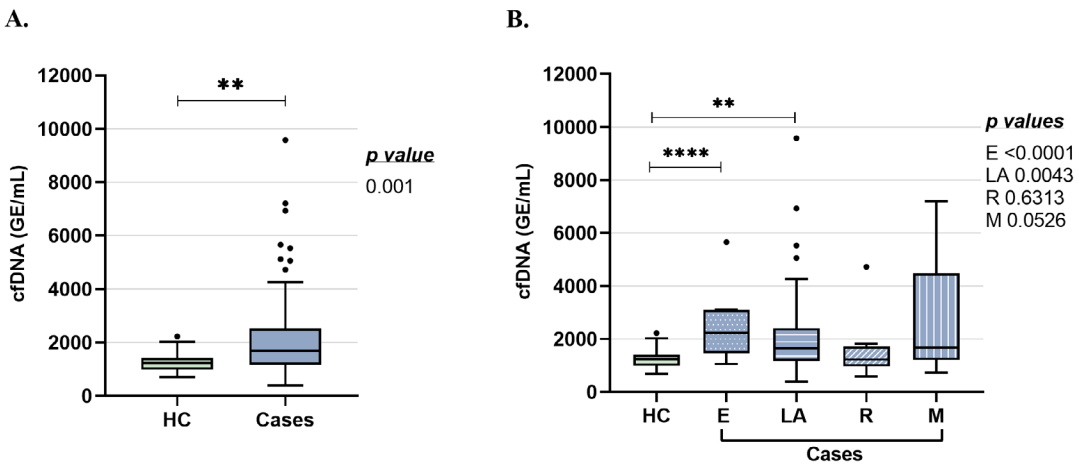


FIGURE 2 | Plasma levels of ccfDNA between healthy controls ($n = 28$) and HNSCC cases at the moment of diagnosis. (A) Box plots depicting the distribution of ccfDNA levels in plasma samples collected from HNSCC patients at diagnosis ($n = 85$) compared to healthy controls ($n = 28$). The box represents the interquartile range (IQR), with the median ccfDNA level indicated by the horizontal line inside the box. The whiskers extend to the smallest and largest values within 1.5 times the IQR from the quartiles, and outliers are shown as individual points. Statistical significance between groups was assessed using the Mann–Whitney *U* test. (B) Box plots comparing the concentrations of plasma ccfDNA between the control group (HC) and the different subgroups of HNSCC patients. Significant differences are indicated by asterisks: $p < 0.01$ (**), $p < 0.0001$ (****). ccfDNA, cell-free DNA; E, early head and neck squamous cell carcinoma; GE/mL, genomic equivalents per millilitre; HC, healthy controls; LA, locally advanced head and neck squamous cell carcinoma; M, de novo metastatic head and neck squamous cell carcinoma; R, relapse head and neck squamous cell carcinoma.

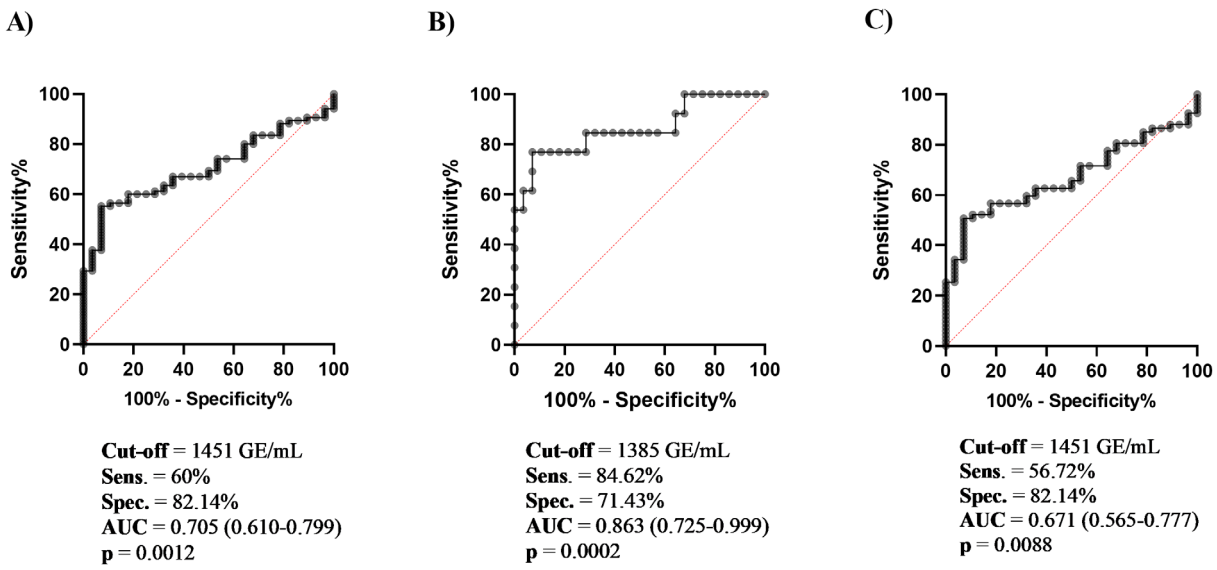


FIGURE 3 | Receiver operating characteristic (ROC) analysis evaluating ccfDNA as a biomarker for distinguishing naïve HNSCC from healthy controls ($n = 28$). (A) ROC curve for ccfDNA distinguishing all HNSCC cases ($n = 85$) from healthy controls. (B) ROC curve for ccfDNA E-HNSCC ($n = 13$) versus healthy controls. (C) ROC curve for ccfDNA advanced HNSCC patients (stage III–IV) ($n = 67$) versus healthy controls. AUC, area under the curve; Sens., sensitivity; Spec., specificity.

administration based on radical chemoradiotherapy (CRT) with or without a previous surgical resection of the primary tumour. No significant differences were observed among the different longitudinal samples (Figure 5). CcfDNA levels in individual patients ($n = 9$) with over four time points follow-up are plotted in Figure S7.

3.6 | Correlation Analysis Between qPCR and Qubit at Baseline

As a secondary objective in our study, we evaluated the consistency between two different ccfDNA quantification methods. To achieve this, we performed two complementary analyses comparing qPCR and Qubit measurements at baseline (S1) of LA-HNSCC patients and in the group of healthy controls. This analysis served us to validate the agreement between the two methods across different populations. First, a correlation analysis (Spearman) demonstrated a strong positive correlation in both groups ($r = 0.8987$, $p < 0.001$ for patients and $r = 0.8871$, $p < 0.001$ for controls), indicating that qPCR and Qubit provide comparable assessments of ccfDNA levels.

Due to the inherent methodological differences between qPCR and Qubit (with qPCR targeting specific genomic regions and Qubit measuring total nucleic acid concentration), a Bland–Altman analysis was performed to assess agreement and potential biases. The Bland–Altman plots revealed a systematic bias between the methods, with Qubit consistently yielding higher ccfDNA measurements. In the healthy control group, the differences between the two methods were generally within the conventional limits of agreement (±2 SD), indicating a constant bias (slope ≈0.12). In contrast, a proportional bias was observed in the LA-HNSCC group, where discrepancies increased as ccfDNA concentrations rose (slope ≠0; 95% CI: 0.32–0.57) (Figure 6).

4 | Discussion

This study underscores the diagnostic potential of ccfDNA while exploring its prognostic value and dynamic changes during and after treatment. By prioritising fluorometry, our goal was to establish a practical, cost-effective, and minimally invasive approach for integrating ccfDNA analysis into routine clinical practice.

First, regarding diagnostic performance, we observed significantly elevated baseline (S1) ccfDNA concentrations in HNSCC patients compared to healthy controls, consistent with previous studies reporting higher median and mean levels in HNSCC using qPCR (Kumar et al. 2017; Verma et al. 2020) and higher mean levels in HNSCC using UV spectrophotometry (Singh et al. 2025) and in OSCC patients using spectrophotometry compared to controls (Desai et al. 2018; Lin et al. 2018). Beyond the head and neck region, similar findings were reported in other cancer types such as lung (Szpechcinski et al. 2015), breast (Khurram et al. 2023), pancreatic (Mattox et al. 2023), colorectal (Wu et al. 2022), stomach, ovary and liver cancers (Cohen et al. 2018) supporting ccfDNA quantification as a potential biomarker for cancer detection.

While higher ccfDNA levels are generally associated with advanced cancer stages (Desai et al. 2018; Huang et al. 2012), our study also showed elevated ccfDNA levels in earlier stages, aligning with a previous finding from Cohen et al. in early-stage ovarian, colorectal and oesophageal cancers (Cohen et al. 2018). One possible explanation is that early-stage tumours might induce systemic inflammatory responses, leading to increased ccfDNA release from both tumour and non-tumour cells (Turabi et al. 2024). These results contrast with the usual trend, where later-stage tumours tend to release more ccfDNA due to increased tumour burden. Aligning with our results, Kowal-Wisniewska et al., who, using electrophoresis, found no significant differences

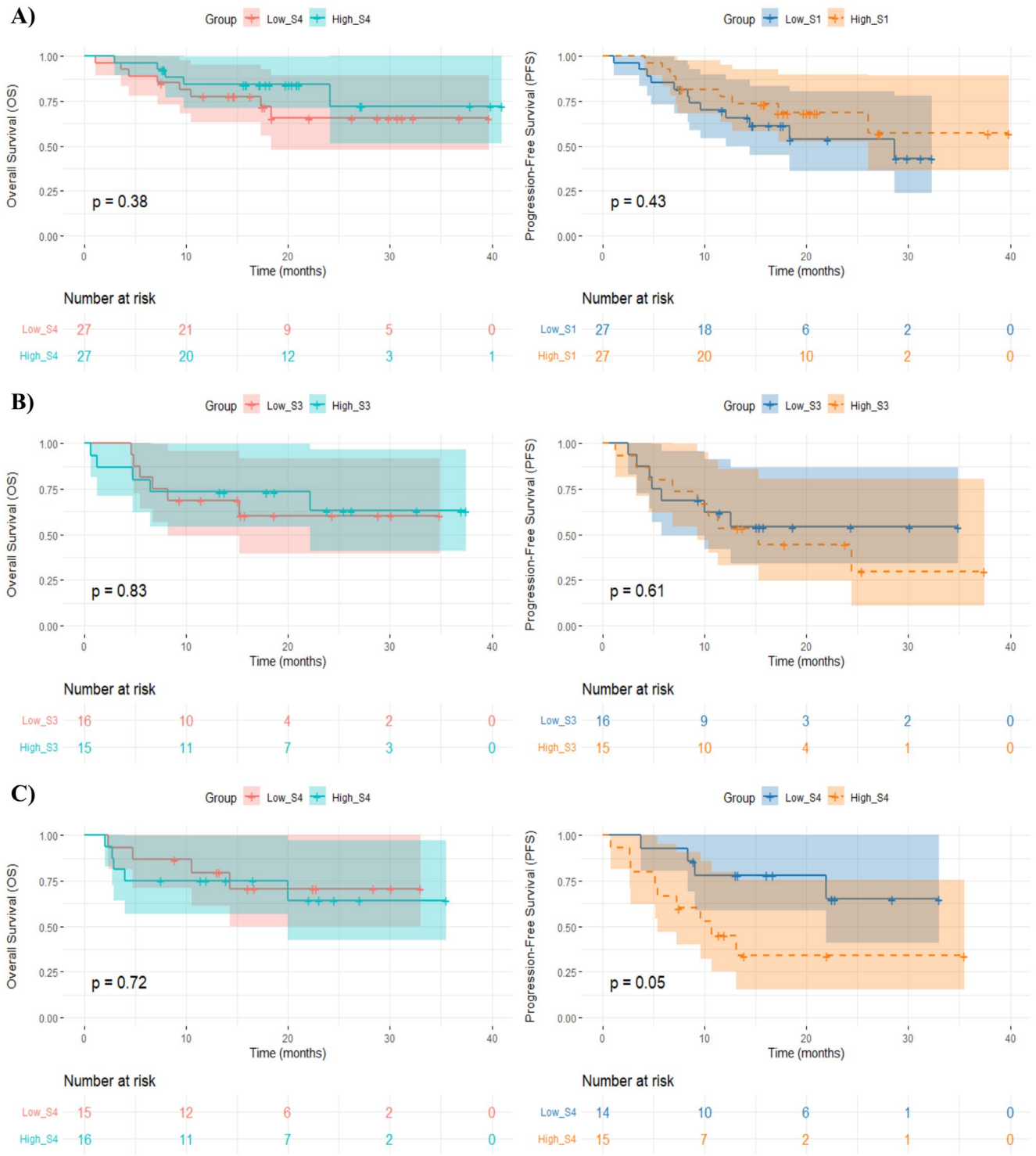
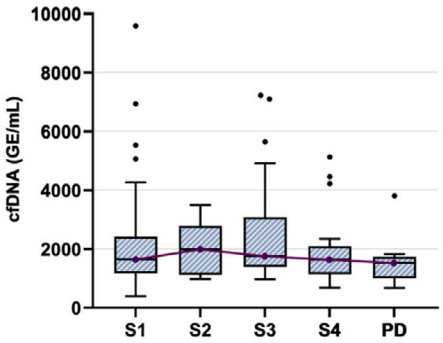


FIGURE 4 | Kaplan–Meier survival analysis plots in patients with LA-HNSCC separated by median ccfDNA levels at different time points (S1, S3 and S4). Kaplan–Meier plots of PFS and OS were generated using a cut-off determined by the median ccfDNA levels obtained at the S1 (A), S3 (B) and S4 (C) timepoints. Tick marks on the curves represent patients whose data were censored, indicating the last time, they were known to be alive (for OS) or free of disease progression (for PFS) during follow-up. ccfDNA, circulating cell-free DNA; OS, overall survival; PFS, progression-free survival.

in median cfDNA concentration between primary and recurrent tumours, arrived at the conclusion that factors other than tumour stage and size might influence the amount of cfDNA released into circulation (Kowal-Wisniewska et al. 2024).

In our study, older patients (≥ 65 years) exhibited higher ccfDNA levels compared to younger patients, a trend also observed in previous studies (Cicchillitti et al. 2017; Kowal-Wisniewska et al. 2024; Mazurek et al. 2016), which suggests that ccfDNA levels tend to increase with age. However, other factors such as intense exercise (Tug et al. 2017), sepsis (Dennhardt et al. 2024), tissue injury or inflammation (Fatouros et al. 2010; Kananen et al. 2023) can influence ccfDNA levels, reducing their specificity for cancer detection.



Statistic	S1	S2	S3	S4	PD
n	**54**	**5**	**31**	**29**	**12**
Min.	389.1	973.6	972.7	680.7	675.8
Q1	1168	1119	1385	1134	1002
Median	**1641**	**1985**	**1758**	**1638**	**1527**
Q3	2416	2780	3076	2085	1740
Max.	9576	3485	7212	5121	3803
Range	9187	2511	6239	4440	3127

FIGURE 5 | Longitudinal plasma ccfDNA levels from baseline up to the moment of progressive disease (PD) for LA-HNSCC cases. Boxplots representing ccfDNA levels at each time point of analysis (from S1 to PD) ($p = 0.334$). The descriptive statistics of samples collected for each time point appear on the right. The test for comparison of median between groups is done by the non-parametric Wilcoxon test. Max., maximum; Min., minimum; PD, progressive disease.

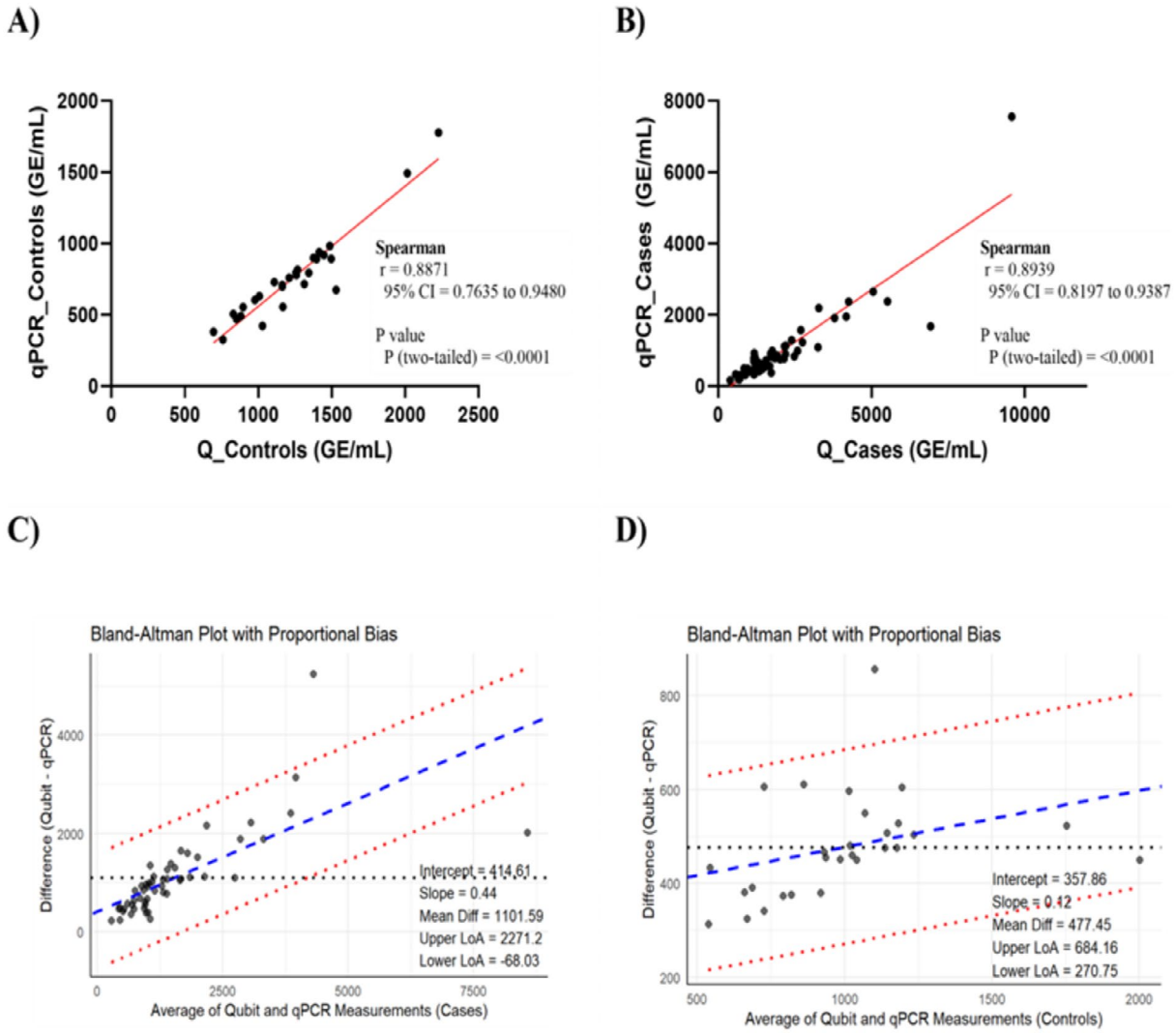


FIGURE 6 | Correlation between qPCR and Qubit methods for ccfDNA quantification. (A, B) Spearman correlation analysis demonstrates a strong positive correlation between qPCR and Qubit measurements in LA-HNSCC patients (A) and healthy controls (B) at baseline (S1) ($r = 0.8987$ for patients and $r = 0.8871$ for controls; $p < 0.0001$ for both). (C, D) Bland–Altman plots for LA-HNSCC patients (C) and healthy controls (D).

While previous studies have reported higher ccfDNA levels in advanced disease stages, such as stage IV tumours or N2-3 nodal disease (Mazurek et al. 2016), we found no differences analysing the correlation between ccfDNA levels and clinicopathological features such as tumour stage, size, or location. This discrepancy may be due to the higher ccfDNA levels that we observed

in patients with stage I–II disease, who tend to have smaller tumours without nodal involvement. Consistent with our findings, other studies have also reported no significant correlation between ccfDNA concentration and disease stage (Kowal-Wisniewska et al. 2024; Shukla et al. 2013) or tumour location (Coulet et al. 2000; Kowal-Wisniewska et al. 2024).

The diagnostic performance of ccfDNA quantification in our study indicates moderate discriminatory power between HNSCC patients and controls (AUC = 0.705). However, it is important to emphasize that no liquid biopsy-based biomarker has yet been approved for improving HNSCC management. In contrast, in malignancies such as lung or colorectal cancer, several FDA-approved liquid biopsy assays are already being used for cancer diagnosis, treatment selection and response monitoring (Coronado et al. 2024; Lanman et al. 2015; Woodhouse et al. 2020). Nowadays, diagnosis of HNSCC still relies primarily on visual inspection, physical examination (e.g., endoscopy) or imaging studies followed by histopathological analysis. However, the intrinsic anatomical complexity of the head and neck region, the absence of specific early symptoms and interobserver variability in clinical evaluation supports the urgent need to develop new strategies for improving HNC management. In this context, even a modestly sensitive non-invasive biomarker such as ccfDNA may offer meaningful clinical utility in a non-invasive manner. Notably, in our subgroup of early-stage HNSCC patients, ccfDNA levels demonstrated improved diagnostic performance, supporting its potential as a valuable tool for optimizing early detection and improving prognosis. Compared with other emerging biomarkers that are being under investigation, such as circulating miRNAs, exosomal RNA, proteomic or metabolomic profiles, ccfDNA concentration offers advantages such as being easily quantifiable with standardized methods (Till et al. 2021) and the potential for additional applications beyond total concentration measurement (including mutation profiling, methylation patterns, or fragmentomics), which might facilitate its integration into daily clinical practice and enhance its value as clinical biomarker. However, as ccfDNA is not cancer-specific, combining it with other biomarkers through integrated multi-omic liquid biopsy approaches could enhance diagnostic accuracy and provide a more comprehensive molecular characterisation of HNSCC. In this regard, we have recently reported the potential of cell-free DNA integrity as a complementary biomarker for cancer detection (Rodríguez-Ces et al. 2024).

While ccfDNA quantification provides a general measure of circulating DNA levels, cell-free DNA integrity indices, usually calculated as ratios of DNA fragment sizes, may offer tumour-specific insights by capturing processes like apoptosis and necrosis. Combining ccfDNA quantification with cell-free DNA integrity could significantly improve the diagnostic accuracy of liquid biopsies as demonstrated in a recent article regarding early non-small cell lung cancer diagnosis (Ren et al. 2024).

We also explored the prognostic potential of ccfDNA, which has already been established in other cancers (Cargnin et al. 2017; Carrasco et al. 2022; Fernandez-Garcia et al. 2019). In our study, ccfDNA levels were analysed at three timepoints (S1, S3 and S4), and patients were stratified into 'High' and 'Low' groups based on the median ccfDNA concentration. Elevated ccfDNA levels at S4 were associated with lower PFS (approximately 9.63 months) compared to patients with low ccfDNA levels (16.37 months). This highlights the potential utility of ccfDNA kinetics as a late-stage risk stratification tool. Further validation in larger cohorts is necessary to confirm these findings and better understand the prognostic implications of ccfDNA dynamics in LA-HNSCC.

In our LA-HNSCC cohort, considerable inter-patient variability was observed in ccfDNA kinetics. Most patients showed elevated ccfDNA levels after CRT treatment (S3) compared to baseline, consistent with previous studies linking this to increased cellular activity and inflammation following radiotherapy (Koukourakis et al. 2023; Uchibori et al. 2025). However, a subset of patients demonstrated stabilization or even a decrease in ccfDNA levels at S4. This decrease may reflect tumour reduction typically seen 4–8 weeks after radiotherapy, as defined by the RECIST 1.1 guidelines (Kageyama et al. 2018) and described in other articles in the field (Kowal-Wisniewska et al. 2024). Despite these fluctuations, no clear correlation was found between ccfDNA levels at S4 and imaging-based response evaluations or progression risk. In patients with PD, ccfDNA levels either remained elevated or showed recurring peaks after S4, suggesting dynamic changes during disease monitoring. These findings align with those of Uchibori et al. (Uchibori et al. 2025), who reported considerable variability in ccfDNA kinetics in OSCC patients using fluorometry with increases and decreases in the total cfDNA concentration in patients with and without recurrence or metastasis. These dynamic patterns suggest that total ccfDNA concentration changes may reflect treatment response and disease progression but are also influenced by factors such as tumour burden, treatment modality and timing of sample collection.

Unfortunately, the absence of sequential samples between S4 and PD limited our ability to track ccfDNA changes during this critical interval. Additionally, the small cohort size and challenges in obtaining follow-up samples further constrained our analysis. Nonetheless, these findings indicate that ccfDNA kinetics may hold promise as a real-time biomarker for predicting PD in LA-HNSCC. Lastly, our correlation analysis between qPCR and Qubit showed strong agreement for ccfDNA quantification, aligning with previous studies in cancer patients (Ponti et al. 2018; Till et al. 2021). We found higher ccfDNA concentrations with Qubit than qPCR, with a proportional bias in the LA-HNSCC group, where differences increased as ccfDNA levels rose. This discrepancy may stem from qPCR's high specificity versus Qubit's advantages, such as lower cost, faster processing and the ability to measure total ccfDNA without specific genomic targets. These benefits make fluorometry a practical, cost-effective option for routine ccfDNA quantification in clinical settings, particularly for distinguishing between high and low ccfDNA levels to help patient stratification. Additionally, these findings highlight the importance of considering regression-based adjustments when comparing quantification methods, particularly in clinical scenarios where ccfDNA concentrations may vary widely.

This study is not without limitations. While our overall cohort ($n = 85$) provides reasonable power, some subgroups present a small sample size (e.g., M-HNSCC, R-HNSCC), which limited the ability to detect significant associations and may have influenced the robustness of our findings. Therefore, the

analyses within these subgroups should be regarded as exploratory. Future studies with larger, adequately powered cohorts will be necessary to validate these preliminary observations. Additionally, incorporating more frequent longitudinal sampling during HNSCC follow-up, particularly between S4 and PD, would enhance our understanding of cfDNA kinetics during this critical period, providing clearer insights into its relationship with PD. Finally, it is important to note that all participants were Caucasians, which may limit the generalizability of our findings. Including more ethnically diverse populations in the future research will be essential to better evaluate the applicability of ccfDNA across different genetic backgrounds and clinical scenarios.

Despite these limitations, our study offers several strengths and contributes novel insights that help advance in the field of ccfDNA research in HNSCC. Firstly, it includes a relatively large and well-characterized cohort of 85 patients encompassing all disease stages and several tumour subsites, offering a more comprehensive overview of the role of ccfDNA across the disease spectrum. This broad inclusion enables us to capture the heterogeneity of HNSCC, which often overlooked in studies focused on specific stages or locations. We also provide a comprehensive analysis of ccfDNA kinetics in LA-HNSCC patients at multiple clinically relevant timepoints. In contrast to earlier studies that lacked follow-up data, this longitudinal approach sheds light on how ccfDNA levels fluctuate over time, offering the initial evidence of its potential utility as a dynamic biomarker for disease monitoring and treatment response in HNSCC. To ensure robust and reliable measurements, we employed two quantification methods, Qubit and qPCR, emphasising the advantages of fluorometry as a simple, cost-effective option for routine clinical implementation. Finally, the multicentric, prospective design of our study further strengthens its generalizability across diverse patient populations, addressing limitations of prior single-centre studies.

5 | Conclusion

Our study provides evidence of the potential value of fluorometric ccfDNA quantification as a diagnostic, prognostic and monitoring biomarker for HNSCC. However, further well-designed studies must be carried out to validate these findings to enhance the clinical utility of ccfDNA as a biomarker for HNSCC management.

Author Contributions

Ana María Rodríguez-Ces: conceptualization, investigation, writing – original draft, visualization, methodology, formal analysis, software, data curation, validation, resources, writing – review and editing. **Óscar Rapado-González:** conceptualization, data curation, investigation, supervision, writing – original draft, writing – review and editing, methodology. **Santiago Aguín-Losada:** supervision, writing – review and editing. **Inés Formoso-García:** supervision, writing – review and editing. **José Luís López-Cedrún:** supervision, writing – review and editing. **Gabriel Triana-Martínez:** supervision, writing – review and editing. **Rafael López-López:** supervision. **María Mercedes Suárez-Cunqueiro:** conceptualization, funding acquisition, project administration, writing – original draft, writing – review and editing, supervision.

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Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

All authors have read the journal's policy on disclosure of potential conflicts of interest and report the following: Rafael López-López reported Nasasbiotech during the conduct of the study; received grants and personal fees from Roche and Merck, personal fees from AstraZeneca, Pharmamar, Leo and Bayer and personal fees and non-financial support from BMS outside the submitted work. The rest of the authors have nothing to disclose. All authors have read the journal's authorship agreement, and the manuscript has been reviewed by and approved by all named authors.

Data Availability Statement

All data generated or analysed during this study are included in this published article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.