Lead Time to Recurrence After Posttreatment Plasma and Saliva HPV DNA Testing in Patients With Low-Risk HPV Oropharynx Cancer

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TITLE PAGE

Article Title: Post Treatment Plasma and Saliva HPV DNA Testing Provides Lead Time to Recurrence in Low-risk HPV Oropharynx Cancer

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Key Points:

Question: What is the effectiveness of utilizing plasma and salivary Human Papilloma Virus (HPV) DNA to define risk for recurrence in patients with HPV oropharynx cancer?

Findings: In this cohort study of 233 patients from a prospective, randomized controlled trial, the sensitivity and specificity of plasma and salivary rinse HPV DNA assays for detecting recurrence were 65% and 87% respectively. The mean lead time from positive test to recurrence date was 122 days.

Meaning: Post treatment presence of HPV DNA in plasma and salivary rinses is associated with recurrence and offers an opportunity for earlier detection of recurrence.

ABSTRACT

Importance: Head and neck squamous cell carcinoma (HNSCC) is a highly lethal cancer that is often associated with human papilloma virus (HPV). Recent studies show promise in the use of HPV DNA detection in salivary rinses and plasma as a predictor of clinical behavior in Human Papilloma Virus Positive Oropharynx cancer (HPVOPC). However, the utility of plasma and salivary HPV DNA detection in defining risk for recurrence in the context of a prospective, phase III, clinical trial coupled to standardized clinical surveillance has not been reported.

Objective: To identify patients with low-risk HPVOPC at risk for recurrence by detection of HPV16 DNA in plasma and salivary rinses.

Design: Patients were recruited from the De-ESCALaTE HPV Trial, an open-label, phase III randomized controlled trial. Patients were assayed for the presence of HPV16 DNA in plasma and salivary rinse via a quantitative PCR based assay.

Setting: Patients were recruited from 32 head and neck treatment centers in Ireland (n=1), the Netherlands (n=1), and the UK (n=30).

Participants: 233 low-risk patients from a randomized controlled trial of cetuximab vs cisplatin for HPVOPC

Main Outcomes and Measures: Assay results were correlated with risk of recurrence and lead time from HPV16 DNA detection to recurrence.

Results: 1040 salivary or blood samples were taken over the course of the study. With a median follow up of 760 days, the sensitivity and specificity of combined plasma and salivary rinse HPV DNA assays for detecting recurrence were 65% and 87% respectively. There was a median lead time of positive test to event/recurrence date of 19 days and mean of 122 days

Conclusion and Relevance:

In the setting of a randomized, prospective, phase III trial for low-risk patients with HPVOPC, post treatment presence of HPV DNA in plasma and salivary rinses is associated with recurrence, and a lead time between test positivity and clinical recurrence offers an opportunity for earlier detection of recurrence.

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INTRODUCTION

HPV positive oropharynx cancer (HPVOPC) has been traditionally associated with sexual exposure, with HPV16 as the predominant subtype.[1,2] Poor vaccine compliance and a long latency period drives a continued high incidence of HPVOPC in the US and other countries.[3-6] HPVOPC is associated with a 20% mortality at three years and recurrence rates of 10-25%, despite combined modality therapy, [3,7-10,11,12] The current National Comprehensive Cancer Network (NCCN) guidelines for post treatment surveillance for HPVOPC recommend post-treatment baseline imaging within 3- 6 months of treatment.[13] However, routine clinical surveillance does not often identify asymptomatic disease recurrence.[14]

Recent studies show promise in the use of HPV DNA detection in salivary rinses and plasma as a predictor of clinical behavior in HPVOPC based on heterogenous cohorts.[15-21] Cao et al. found that HPV DNA in plasma was found in 65% of the HPVOPC patients (n=40) and became undetectable with disease resolution.[22] Chera et al. were able to define a sequential, multiple testing threshold for HPV presence in post treatment plasma samples that defined a low recurrence risk, and a subsequent study using the same assay in a larger cohort showed a strong association of test status with recurrence.[15,23] Similarly, other studies have shown an association between overall survival and post treatment HPV-positive status in either saliva or plasma.[24] However, the utility of plasma and salivary HPV DNA detection in defining risk for recurrence in the context of a clinical trial coupled to standardized clinical surveillance has not been reported. We determined the sensitivity, specificity, and lead time of post treatment plasma and salivary HPV16 DNA for detection of recurrence in De-ESCALaTE, a randomized, prospective, multi-institutional, phase III trial for low-risk patients with HPVOPC.

MATERIALS AND METHODS

Study Design and Cohort

Patients were recruited from an open-label, phase III randomized controlled trial that examined the toxicity and efficacy of 1) cetuximab and radiotherapy compared to 2) cisplatin and radiotherapy in previously untreated low-risk patients with HPVOPC (AJCC VII Stage III-IVa oropharyngeal SCC tumors, excluding patients with both N2b, N2c or N3 nodal disease and more than 10 Pack Years of smoking).[12] All patients included in this study had biopsy proven HPVOPC that was confirmed to be p16 positive on immunostaining. The 334 patients enrolled in De-ESCALaTE were offered enrollment in this sub-study for plasma and salivary rinse harvest at pretreatment, 3 and 12 month post-treatment timepoints. (Figure 1). A total of 233 patients underwent a sample harvest at some point during the study. 207 patients underwent sample harvest prior to treatment start and 204 patients completed at least one salivary rinse or plasma assay after treatment completion. Exclusion criteria included an assay date that was not recorded or incorrect (n=2), assay date that was after a documented recurrence date (n=9), and final pathologic specimen that was negative for HPVDNA (n=11). Patients were assessed for treatment response 12 weeks after radiotherapy completion by clinical examination and by CT, MRI, or PET-CT scan. Follow-up consisted of clinical examination, monthly in the first year and every 2 months in the second year, for at least 24 months after treatment completion. All recurrences were confirmed pathologically.

All patients gave written informed consent for their tissue and patient data to be used in additional research. Patients were assigned a unique identifier such that patients were not identifiable to members of the research team. Blood samples were collected into 10mls EDTA tubes. Upon receipt, each tube was processed according to Institute of Head and Neck Studies and Education (InHANSE) standard protocols. Briefly, they were centrifuged, and the plasma aliquoted and stored at -80^{o}C in cryovials. Salivary rinse samples were collected following the patients having

gargled 8ml PBS and it was subsequently diluted 1:1 in 70% ethanol. Clinical data are stored/archived securely on central servers hosted by the University of Birmingham and at the Warwick Clinical Trials Unit, University of Warwick.

HPV DNA Assay

A PCR based test for HPV16 DNA in plasma and salivary rinses was performed on linked, deidentified samples in a CLIA certified laboratory at the Johns Hopkins Clinical Medical Microbiology Laboratory as previously reported.[25-31] Assays were performed on 1 ml of plasma and a 0.5 ml aliquot of saliva rinse representing half of the centrifuged cell pellet from an 8 ml saline oral rinse, using primer probe combinations targeting the E5L2 and E1 regions, as well as the E7 region.[21-26] Briefly, nucleic acid from plasma and oral rinse samples were extracted using the easyMag instrument (bioMerieux), input and elution volumes were 500 and 50 µL, and quantitative real-time PCR amplification was performed on a 7500 Real-Time PCR System for 3 genomic regions of HPV16. Each reaction consisted of TaqMan 2X PCR Master Mix (ThermoFisher), 450 nM of forward and reverse primer, 200 nM of FAM-labeled probe, and dH2O (40µL) to which 10 µL of template was added. Quantification standards were prepared by cloning HPV16 amplicons from each of the 3 regions into a pCR®2.1-TOPO® plasmid vector (Invitrogen/ThermoFisher Scientific). Serial 10-fold dilutions of plasmid from 7.0 to 1.0 log10 copies/reaction were included with each assay and used to establish a standard curve. Data was expressed as HPV16 DNA copies/mL for each genomic region.

Statistical Analysis

For descriptive statistics, means, medians, and frequencies were calculated as appropriate. Kaplan-Meier plots and the Cox-proportional hazard regressions were used to investigate associations of the

assay with recurrence/persistence free survival. In the Cox-proportional hazard regressions, the HPV DNA assay result was included as a time varying predictor (i.e., a subject enters the risk set only after an assay was taken). Potential confounders were selected based on known prognostic factors and included age, gender, smoking status, study arm, stage, and subsite. For these confounders, we first fit a univariate model for each variable and identified confounders significant at the level of 0.1. No variables were retained after this selection process for the primary analysis. Lead time was calculated from date of assay completion to date of clinically or radiographically detected recurrence. Recurrence was defined as biopsy-proven return of disease after completion of post treatment imaging showing remission. Recurrence could be locoregional or distant, or both. All tests and confidence intervals were two-sided and significance was defined as a p-value <0.05. All analysis was carried out in R (4.1.2).

RESULTS

Patient Characteristics

Between November 2012 and October 2016, 233 patients completed salivary rinse or blood samples with a total of 1040 samples completed (Table 1). The mean age of all patients with a completed assay was 57 years old; 81% of the patients were men and 46% were either current or past smokers; 65% had tonsil subsite primaries and 33% had base of tongue primaries; 16% were stage III disease, 84% of patients were stage IVa disease. 28 patients had evidence of recurrence during follow up; 6 patients had persistent disease; and 4 patients died.

Pretreatment Evaluation

Pretreatment performance of HPV16 DNA detection in plasma and salivary rinse was first determined. 188/207 patients with biopsy proven HPVOPC had positive tests (91%) prior to treatment start (Supplementary Table 1), with a sensitivity of 87% and 75% for plasma and salivary

rinse, respectively. There were no differences in survival based on pretreatment results (p=0.96) (Supplementary Figure 1).

Longitudinal HPV16 DNA Monitoring

There were 204 patients with HPVOPC with total of 675 salivary rinse and/or plasma assays completed after treatment. 39 patients (19.1%) had positive assays post treatment completion. Of these 39 patients, 15 patients (38.5%) had evidence of recurrence at any point after assay date. There was a median lead time of positive test to event/recurrence date of 19 days and mean of 122 days (Range: 0-536 days). The sensitivity and specificity value of combined salivary rinse and plasma for detecting recurrence were 65% and 87% respectively. Analysis of plasma assays (n=201) alone had a higher specificity (98%) but lower sensitivity for detecting early recurrences (35%). (Figure 2, Table 2). Probability of recurrence was independently associated with post-treatment positive plasma and or salivary rinse samples in a cox model (HR 13.15; 95% CI 4.83, 35.78) (P = <0.001) (Figure 3).

Secondary Analyses

We then restricted our analysis to patients that had positive plasma or saliva HPV16 DNA assays prior to treatment initiation, with the hypothesis that these patients have a demonstrated detectable HPV16 subtype of their oropharynx cancer. Of 163 patients that had positive assays prior to treatment initiation and completed at least one post treatment assay, 31 patients (19.0%) had a positive assay post treatment completion. 12 of these 31 patients (38.7%) had evidence of recurrence, with a median lead time of 31 days, mean lead time of 140 days and a sensitivity and specificity of 75% and 87%, respectively. Analysis of plasma alone (n=161) had a higher specificity (98%) but lower sensitivity for detecting early recurrences (38%). Recurrence was similarly associated with a post treatment positive assay at any point by Cox regression (Supplementary Figure 2 HR 17.12; 95% CI 4.85, 60.49) (P = <0.001).

Copy number variation

As a post hoc analysis, we analyzed assayed quantitative HPV DNA copy numbers in plasma and salivary rinse to determine if a cutoff threshold to define a positive test could optimize test performance. There were large ranges in copy number in positive plasma assays (median 130, range [14,23000]) and salivary rinse assays (median: 63.5, range [3.3,39000]), and thresholds were set at 15.5 copies/ml for plasma assays and 8.45 copies/ml for salivary rinse assays to maximize AUC with a sensitivity and specificity at 65% and 90% for detection of recurrence. Thus, there were only modest improvement in specificity by instituting a copy number threshold for assay positivity. Of note, quantitative measures of plasma HPV copy number (log transformed) were significantly associated with the recurrence free survival (P=<0.001, HR= 4.78; 95% CI 3.49,6.56).

DISCUSSION

This is the first study to define the utility of HPV DNA plasma and saliva testing in the setting of a prospective, phase III, multi-institutional trial for patients with HPVOPC. This demonstrates that HPV16 DNA in plasma and saliva is independently associated with subsequent recurrence. As recurrent HPVOPC may be effectively treated,[32] a testing strategy in low-risk HPVOPC may potentially improve the clinical outcomes of patients by earlier detection of recurrence by providing a window of opportunity for clinical intervention, including early imaging or potential intensification of therapy. However, negative or positive testing did not absolutely exclude or assure HPVOPC recurrence, respectively, even in the setting of extended surveillance in a prospective clinical trial. Taken together, these data indicate that while HPV DNA presence in post treatment plasma and saliva is an independent risk factor for recurrence with a robust lead time, HPV DNA plasma and saliva testing should not be used alone to confirm or exclude suspected recurrence. Notably, these data are based on a trial for low-risk HPVOPC, and performance of HPV

DNA testing in plasma and saliva in higher-risk locoregionally advanced and recurrent/metastatic HPVOPC patients will undoubtedly display different test performance based on difference in risk of recurrence, as well as potential differences in underlying biology of HPV DNA shedding in plasma and salivary rinse compartments.

Our findings of high specificity and moderate sensitivity for HPVDNA biomarkers in detecting recurrences early in HPVOPC is consistent with many prior studies.[17,24,33] However, these studies were limited by small sample sizes, limited follow up time and some studies only looked at salivary rinse samples.[17,24] Multiple recent studies have looked at using HPVDNA to detect malignancies and treatment response as a potential indication for HPVDNA assays.[19-21,34,35] Siravegna et al and Damerla et al recently completed observational studies using HPV DNA for diagnosis of HPV positive oropharynx cancer. While these studies demonstrate a high sensitivity and specificity, these studies were limited to patients with confirmed malignancy and did not analyze using HPV DNA as a tool for detection of recurrence.[36,37] Most recently, Chera et al published a prospective clinical study using an HPV DNA biomarker in a heterogenous group of non-metastatic HPVOPC, defining two consecutive positive assays with a high positive predictive value (95%) and negative predictive value (100%) in detection of recurrence.[15] We did not find as high a negative predictive value, potentially due to Chera et. al.'s requirement of two consecutive positive assays as well as their much more intensive testing regimen. In their study, patients were tested for HPV DNA every week during treatment and at every post-treatment visit, resulting in 115 patients that were tested with over 1000 assays.

Our findings of a mean lead time from positive assay to detected recurrence was approximately 4 months, which is similar to reported mean lead times in the current literature ranging from 3.9-4.4 months.[15,24] This may suggest that some patients begin to develop a detectable

viral load approximately 3-4 months prior to clinical evidence of disease, though this is difficult to confirm with the current data available. However, these data suggests that a positive HPV DNA test may be used to trigger enhanced surveillance but should not replace standard clinical and radiologic evaluation of disease.

Prior studies have used positive predictive value (PPV) and negative predictive value (NPV) to assure a lack of recurrence and vice versa.[15,23] Most recently, Berger et. al. completed a retrospective clinical case series where they found a high PPV and NPV for their HPV DNA Assay.[23] However, this analysis was limited by the retrospective nature of the study, the lack of staging or treatment information on the cohort, and a short (9 month) median follow up. PPV and NPV are also not solely intrinsic to the test but based on prevalence and characteristics of the studied population, and clinical utility of tests depends on the specific population in which tests for recurrence risk are employed.

A notable limitation to our study was that the original prospective trial used AJCC VII to stage patients. Therefore, our study included AJCC VII Stage III-IVa HPV positive oropharynx squamous cell carcinoma. If restaged using the AJCX VIII updated staging system, 9% of patients would be stage I disease, 73% would be stage II disease, and 18% would be stage III disease.

In general, demonstration of the utility of secondary screening for recurrent HPVOPC faces some inherent challenges. With a recurrence rate for low risk HPVOPC estimated at 9%, and a favorable outcome for treatment of early recurrence, a large sample size is likely needed to provide adequate power to validate the use of a novel biomarker to impact survival from recurrence.[38] However, given the extended lead time of HPV DNA detection prior to recurrence in the context a prospective clinical trial for low-risk HPVOPC, post treatment HPV DNA testing holds promise to provide a mechanism to improve treatment of recurrent disease and ultimate clinical outcome.

CONCLUSION

Post treatment HPV DNA in plasma and salivary rinse assays are associated with risk of recurrence in low-risk HPOPC patients with high specificity and moderate sensitivity in detecting recurrences early in HPVOPC. Patients with positive HPV DNA assays developed a recurrence at a mean of 122 days from assay date, providing a potential window for earlier detection of recurrence. However, a post treatment negative test for HPV16 in plasma and/or saliva does not completely preclude recurrence.

Table I.
Demographic and Clinical Information.

	All Patients (n=233)
Total Number of Assays	1040
Number of Assays taken prior to treatment initiation	
Salivary Rinse	160
Plasma	205
Number of Assays taken post treatment end	
Salivary Rinse	321
Plasma	354
Mean Age	57.01(8.45)
Sex	
Female	45
Male	188
Smoking Status	
No	126
Yes	107
Subsite	
Base of Tongue	78
Tonsil	152
Other	3
Stage	
Stage III	38
Stage IVa	195
T stage	
T1	53
T2	98
T3	42
T4	40
N stage	
N0	17
N1	32
N2	184
Study Arm	
Arm A (RT + cisplatin)	114
Arm B (RT + cetuximab)	119
Clinical Outcomes	
No evidence of death, recurrence or persistence of disease	195
Death	4
Persistence	6
Recurrence	28

Table II.
HPV DNA Sensitivity and Specificity for Detecting Recurrence

	Sensitivity	Specificity
HPV DNA Assay Completed Post Treatment		
- Salivary Rinse + Plasma	0.65	0.87
- Plasma Only	0.35	0.98
- Salivary Rinse Only	0.45	0.87

Supplementary Table I.
Pretreatment HPV DNA Sensitivity

HPV DNA Tests	False Negative for HPVOPC	True Positive for HPVOPC	Sensitivity of HPV DNA Tests in detecting HPVOPC
Salivary Rinse + Plasma	19	188	0.91
Plasma Only	26	179	0.87
Salivary Rinse Only	40	120	0.75

Supplementary Table II
HPV DNA Sensitivity and Specificity for Detecting Recurrence in Patients with Positive HPV DNA Tests Prior to Treatment Completion

Post Treatment HPV DNA Assay	Sensitivity	Specificity
- Salivary Rinse + Plasma	0.75	0.87
- Plasma Only	0.38	0.98
- Salivary Rinse Only	0.50	0.88

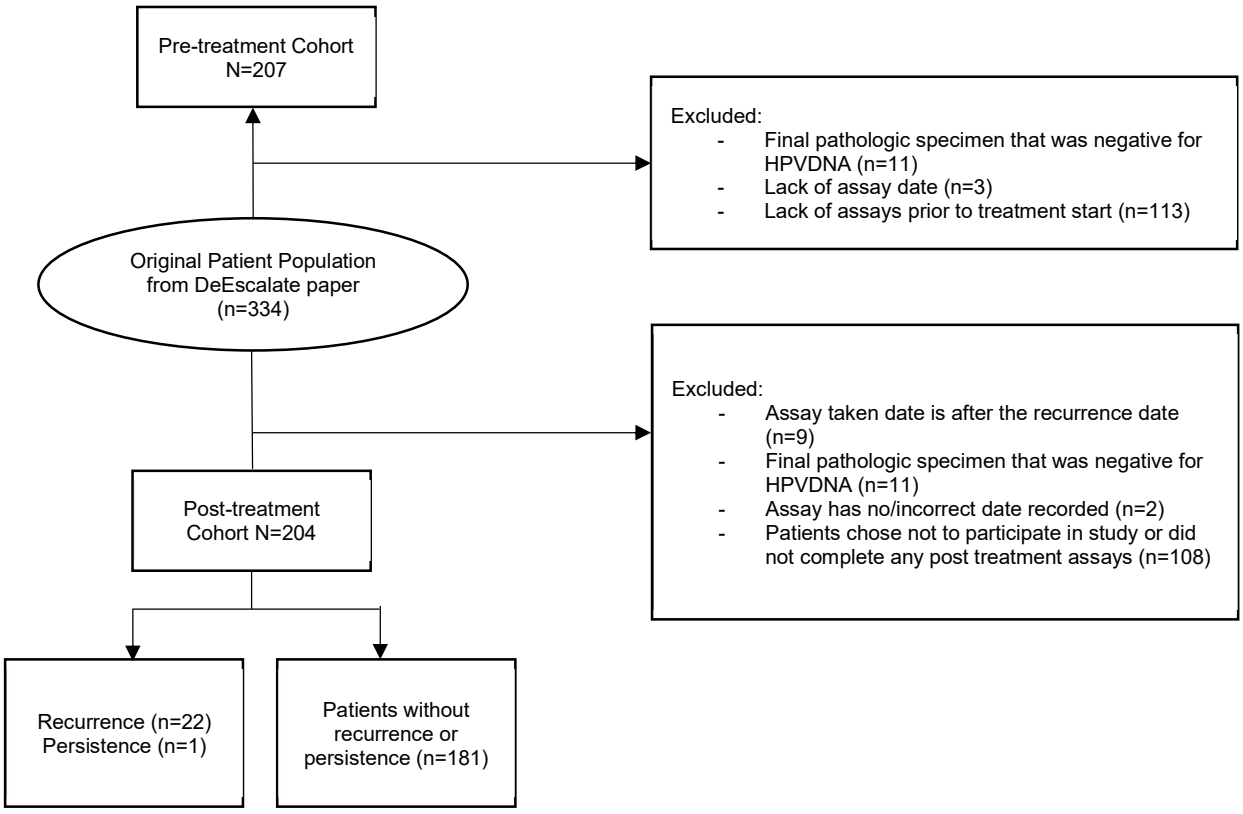


Figure 1: Patient Inclusion and Exclusion Criteria

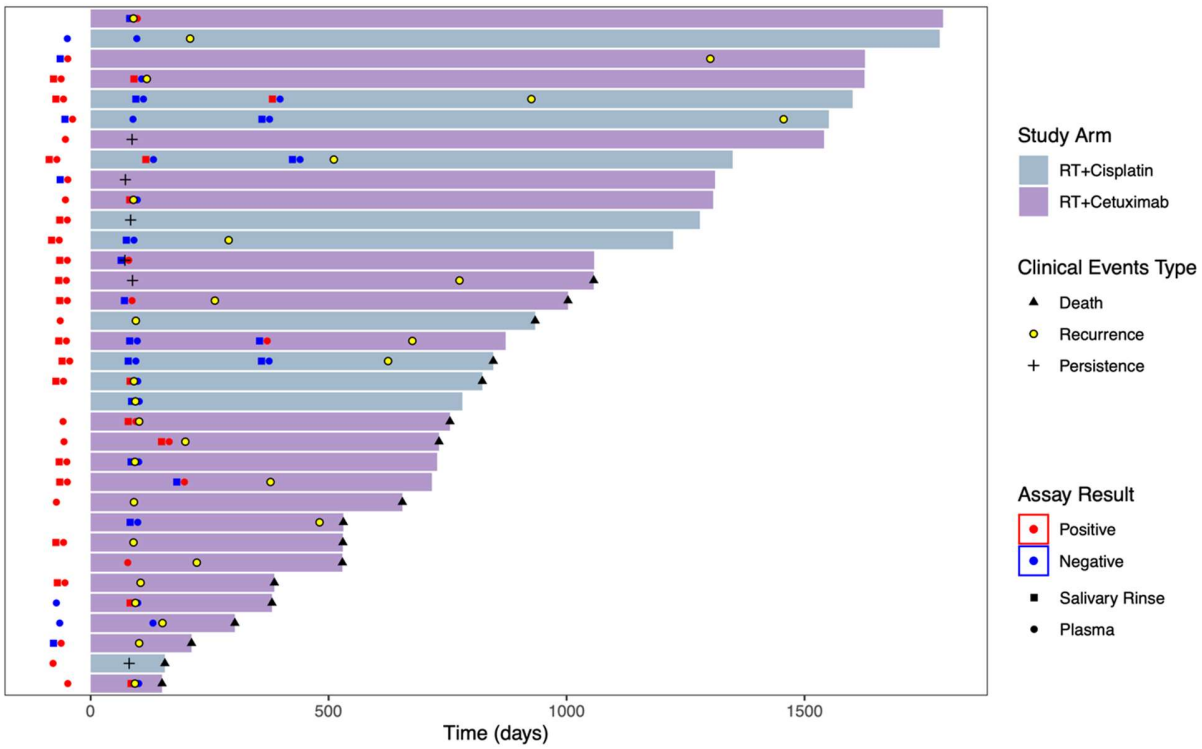


Figure 2: Swimmer's plot for all patients with a recurrence or persistence during the study period with HPV DNA test results

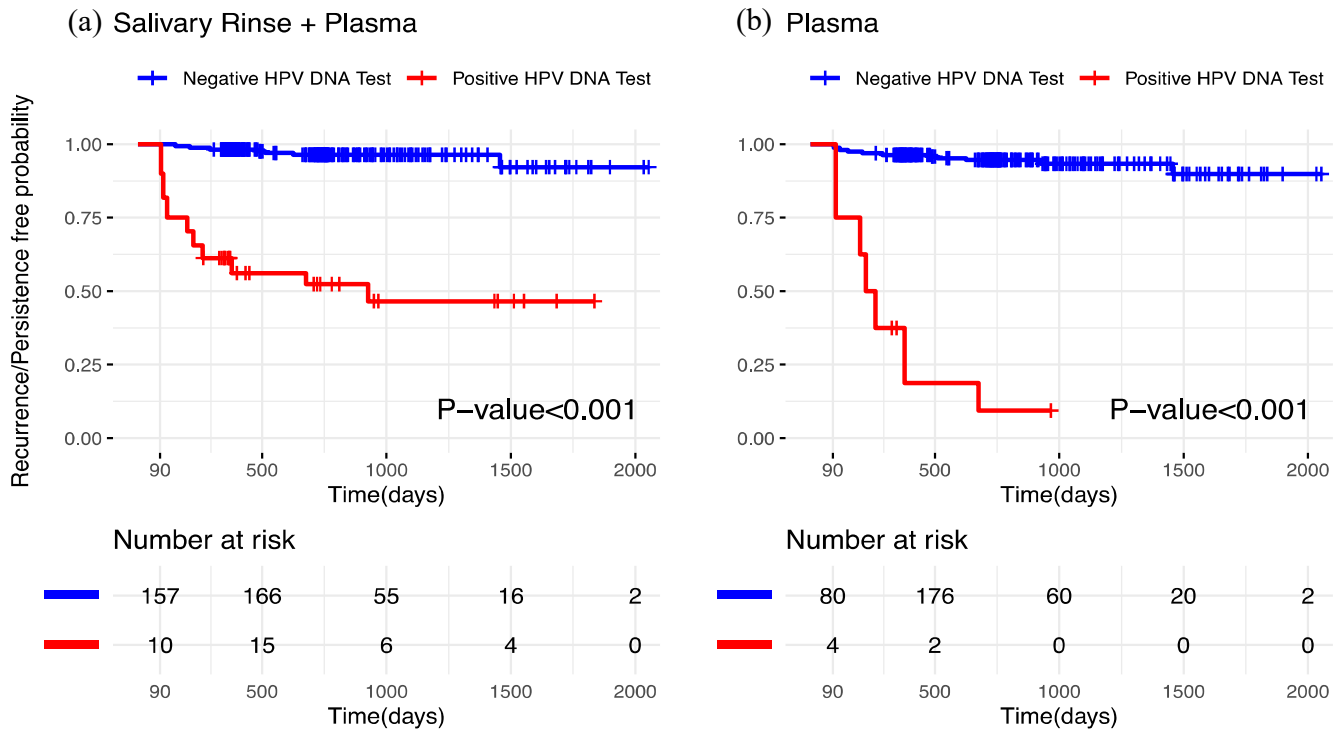
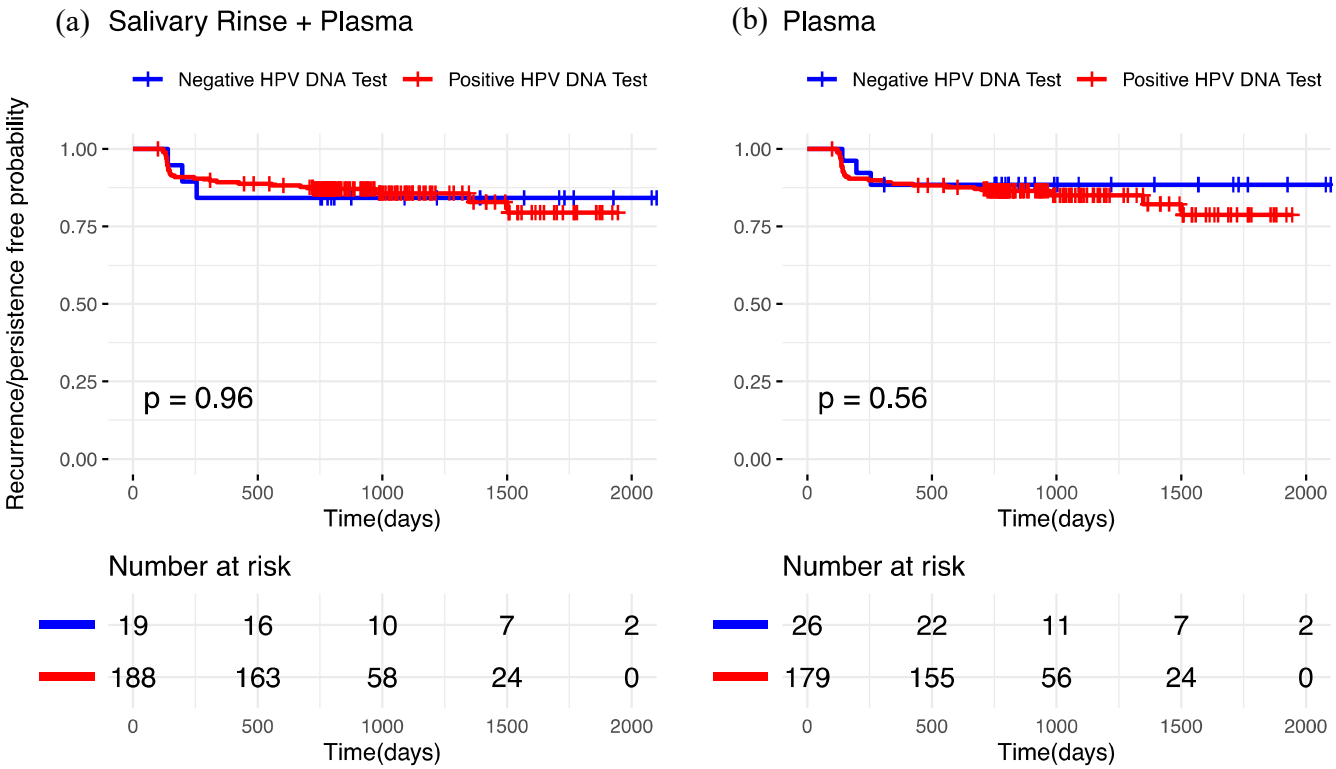
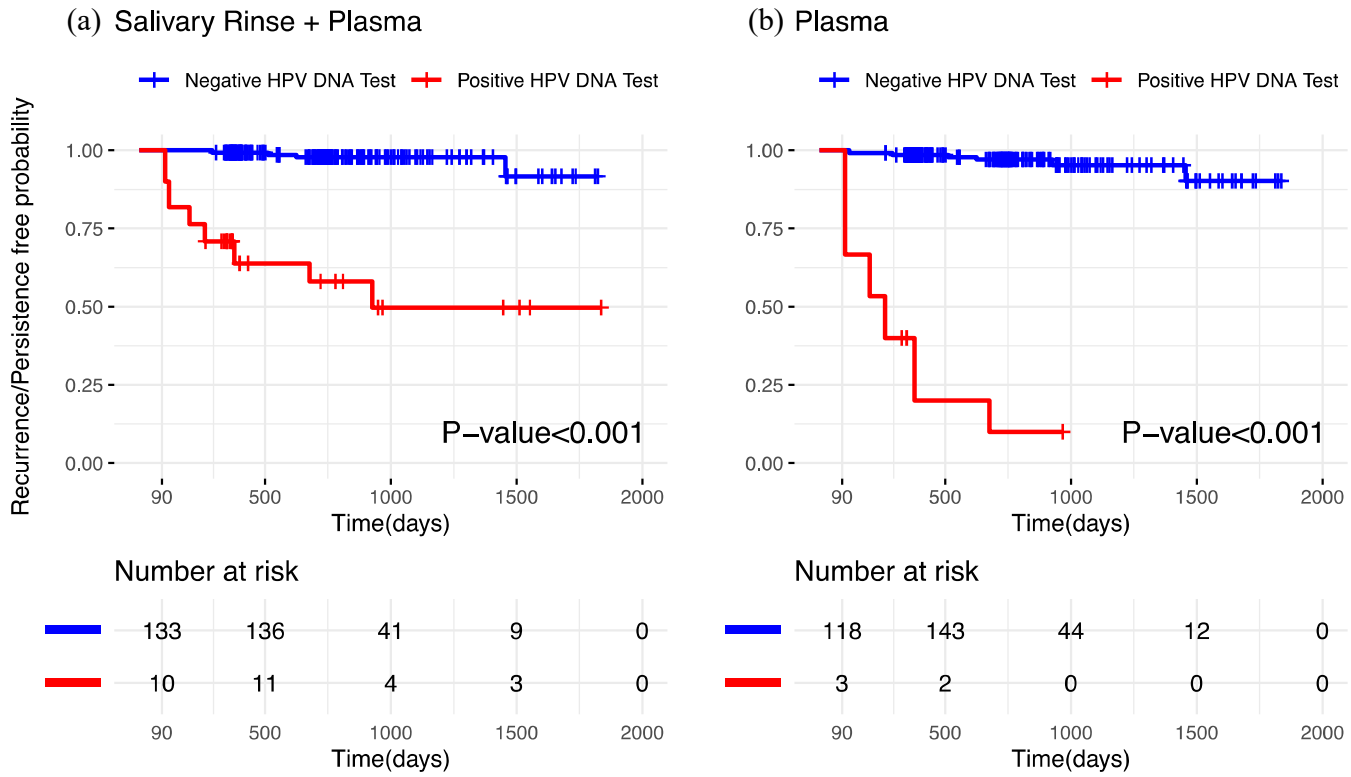


Figure 3: Kaplan Meier curves comparing recurrence free probability in patients with a positive versus negative HPV DNA test in (a) either plasma or salivary rinse assays completed any time after treatment completion and (b) plasma alone assays completed any time after treatment. Univariate Cox regression shows significantly worse rates of recurrence in all groups with a positive assay. Note that since the HPV DNA test result is a time varying predictor, a subject enters the risk set only after an assay is taken, thus the risk set might change as time progress.



Supplementary Figure 1: Kaplan Meier curves looking at recurrence/persistence free probability comparing patients with positive HPV DNA tests prior to treatment initiation with patients with negative HPV DNA tests prior to treatment initiation in (a) either plasma or salivary rinse and (b) plasma alone. Log-rank tests shows no difference in rate of recurrence. Time 0: treatment start



Supplementary Figure 2: Kaplan Meier curves comparing recurrence free probability in patients with a positive versus negative HPV DNA Test after treatment completion restricted to patients with pretreatment positive HPV DNA assays. (a) either plasma or salivary rinse (b) plasma alone. Univariate Cox regression shows significantly worse rates of recurrence in plasma alone and salivary rinse + plasma groups

Figure Legend

Figure 1: Patient Inclusion and Exclusion Criteria

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