# Analysis of HPV, EGFR and Hypoxia Markers and Their Association with Clinical Outcome in Subjects with Locally Advanced Squamous Cell Carcinoma of Head and Neck


***By***

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**[LIFE09201404005]**

**TATA MEMORIAL CENTRE**

**MUMBAI**


***A thesis submitted to***

***The Board of Studies in Life Sciences***

***In partial fulfilment of the requirements***

***for the Degree of***

**DOCTOR OF PHILOSOPHY**

***of***

**HOMI BHABHA NATIONAL INSTITUTE**

**AUGUST, 2021**

# Homi Bhabha National Institute

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As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Ms. Usha Amrutlal Patel entitled "Analysis of HPV, EGFR and Hypoxia Markers and Their Association with Clinical Outcome in Subjects with Locally Advanced Squamous Cell Carcinoma of Head and Neck" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to HBNI.

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**Ms. Usha Amrutlal Patel**

# DECLARATION

I, hereby declare that the investigation presented in the thesis titled *"Analysis of HPV, EGFR and Hypoxia Markers and Their Association with Clinical Outcome in Subjects with Locally Advanced Squamous Cell Carcinoma of Head and Neck"* has been carried out by me under guidance of Dr. Manoj B. Mahimkar. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University**.**

**Ms. Usha Amrutlal Patel**

# List of publication arising from thesis

**Journal**

- Correlation of transcriptionally active human papillomavirus status with the clinical and molecular profiles of head and neck squamous cell carcinomas. Patel U, Mittal N, Rane SU, Patil A, Gera P, Kannan S, Joshi A, Noronha V, Patil VM, Prabhash K, Mahimkar MB. Head Neck. 2021 Jul;43(7):2032-2044. doi: 10.1002/hed.26676. PMID: 33751711.

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- Nimotuzumab-cisplatin-radiation versus cisplatin-radiation in HPV negative oropharyngeal cancer. Noronha V, Patil VM, Joshi A, Mahimkar M, Patel U, Pandey MK, Chandrasekharan A, Dsouza H, Bhattacharjee A, Mahajan A, Sabale N, Agarwal JP, Ghosh-Laskar S, Budrukkar A, D'Cruz AK, Chaturvedi P, Pai PS, Chaukar D, Nair S, Thiagarajan S, Banavali S, Prabhash K. Oncotarget. 2020 Jan 28;11(4):399-408. doi: 10.18632/oncotarget.27443. PMID: 32064043.

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- Prognostic and predictive roles of cancer stem cell markers in head and neck squamous cell carcinoma patients receiving chemoradiotherapy with or without nimotuzumab. Usha Patel, Sadhana Kannan, Swapnil U Rane, Neha Mittal, Poonam Gera, Asawari Patil, Subhakankha Manna, Vishwayani Shejwal, Vanita Noronha, Amit Joshi, Vijay M Patil, Kumar Prabhash, Manoj B Mahimkar. (In communication)

**Conferences:**

**Poster Presentations**

- Poster presentation entitled “Study of correlation between EGFR, hypoxia markers and cancer stem cell markers in locally advanced HNSCC” at 37th IACR Convention, held in Kolkata, India on February 23-25, 2018.

- Poster presentation entitled “Nuclear HIF1α a predictor of survival for nimotuzumab along with CTRT treated HPV negative advanced HNSCC patients” at 4th EACR Conference on Cancer Genomics, held in Cambridge, United Kingdom on 23-26 June, 2019.

**Ms. Usha Amrutlal Patel**

# ACKNOWLEDGEMENTS

I am happy to express my sincere gratitude to all who made my PhD journey possible. First of all, I am infinitely grateful to my research guide **Dr. Manoj B. Mahimkar**, for his constant guidance and support. This doctorate would have not been possible without his valuable suggestions and encouragement throughout my tenure.

I would like to thank **Dr. Sudeep Gupta** (Director, ACTREC), **Dr. Shubhada Chiplunkar** (Former Director, ACTREC), and **Dr. Prasanna Venkatraman** (Deputy Director, CRI-ACTREC) for providing an excellent research atmosphere and infrastructure at ACTREC. I thank my doctoral committee members **Dr. Bhavani Shankar** (DC Chairperson), **Dr. Rukmani Govekar** and **Dr. Sanjeev Waghmare** for giving their critical suggestions during the DC meetings.

I would like to sincerely acknowledge the **Council of Scientific and Industrial Research** (CSIR) for the fellowship to pursue PhD and the **Department of Science and Technology** (DST) for funding my research project. I thank the Director, **Tata Memorial Centre** (Sam Mistry fund) and Director, **Homi Bhabha National Institute** (HBNI) for providing me travel allowance to attend and present my work at EACR conference on Cancer Genomics, held in Cambridge, United Kingdom.

I thank **Dr. Kumar Prabhash** and his team (Head and Neck Unit, Tata Memorial Hospital) for their help with sample collections and providing the patients' follow up data. A special thanks to **Dr. Aparna**, **Dr. Kamesh** and **Reshama sister** for their help and support with patients sample collection. I thank **Dr. Asawari Patil** (Department of Pathology, ACTREC), **Dr. Poonam Gera**

(Biorepository, ACTREC), **Dr. Swapnil Rane** (Department of Pathology, ACTREC), **Dr. Neha Mittal** (Department of Pathology, Tata Memorial Hospital), **Dr. Rajiv Kaushal** (Department of Pathology, Tata Memorial Hospital) and **Dr. Subdha Kane** (Department of Pathology, Tata Memorial Hospital) for helping us with all histopathological grading and immunostaining evaluations. Help from **Mrs. Sadhana Kannan** (Biostatistician, Clinical Research Secretariat, ACTREC) is deeply acknowledged for teaching the different aspects of statistical analysis. I am grateful to **Dr. Gary Clark** for his critical comments and suggestions on our statistical analysis. I acknowledge the constant help from the staff of the Histology Section, ACTERC, especially to late **Mr. V. Sakpal** and **Mr. Dinesh** for all the sectioning of FFPE tissues. I would also like to thank the staff members of the Tumor tissue repository and Pathology Department at Tata Memorial Hospital for providing tumor tissue specimens.

I would like to thank all the members of the Common Instrumentation facility, IT department, the Microscopy facility, the Library facility and the Administrative Department of ACTREC. I would also like to thank the transport facility between ACTREC and Tata Memorial Hospital for making my travel easy and comfortable.

The members of the Mahimkar lab (past and current lab members) have contributed immensely to my personal and professional time at ACTREC. I was fortunate to have very helpful and supportive seniors (**Priyanka and Rasika**). I would like to thank former lab scientific assistant **Mrs. Tanuja Samant** and JRF **Manish** for their help with the project bench work. I also thank project assistant **Nikita** for her help with sample collection. I appreciate the help and support by lab technician RT (**Mr. Ravindra Pawar**). I am very glad to have amazing juniors in the lab- **Mayuri**, **Vaishnavi** and **Zaid** for providing a friendly and joyful atmosphere at work. I am also grateful for their help in numerous ways and accompanying me to have much needed stimulant

(tea) which was a stress buster during the final stages of this Ph.D. I thank all the trainees for the help I received with the bench work- **Rahul**, **Ashvin**, **Pratiksha**, **Prachi**, **Deepak**, **Victor** (Subhakankha Manna) and **Poonam**. I had the pleasure to work with very good senior students and wonderful juniors; they all have contributed to make Mahimkar lab a very lively place to work.

My time at ACTREC was made enjoyable in large part due to all the friends who became a part of my life. I would like to thank all the past and present members of Sorab lab, special thanks to **Sonali**, **Abha**, **Sarika**, **Amol** and **Monica** for keeping the jolly atmosphere in PG room and for all the discussion and debates. I thank the batch of 2014-**Trupti**, **Jyothi**, **Shalini**, **Akash**, **Harish**, **Ajit**, **Saim**, **Mukund**, **Arijit** and **Maitriey** for their pleasant company during all the birthday celebrations, their cooperation and support while conducting NRSM 2017 and all the wonderful and memorable outings. Special thanks to **Trupti**, **Jyothi** and **Shalini** for all the wonderful adventures and sweet memories. I also had a great time with my seniors and juniors at ACTREC and would like to thank them all for being so helpful. I also thank all the patients who participated in the study without which this work would have been impossible.

Last but not the least I would like to thank my parents and all family members for their love and constant encouragement. A special thanks to my uncle (**Mr. Jaswant**), my husband (**Dr. Pravin**) and my brother (**Sanjay**) who have always encouraged me and have supported me immensely throughout this journey.

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## SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student:** Usha Amrutlal Patel
2. **Name of the Constituent Institution:** Tata Memorial Centre, Advanced Centre for Treatment Research and Education in Cancer
3. **Enrolment No. :** LIFE09201404005
4. **Title of the Thesis:** Analysis of HPV, EGFR and Hypoxia Markers and Their Association with Clinical Outcome in Subjects with Locally Advanced Squamous Cell Carcinoma of Head and Neck.
5. **Board of Studies:** Life Science

### Introduction

Head and neck squamous cell carcinomas (HNSCCs) arise in the upper aero-digestive epithelial lining of the head and neck region. HNSCCs comprise a major cancer burden and are a leading cause of mortality in many regions of the world including India (1, 2). The major risk factors associated with the disease are tobacco and/or alcohol abuse and infection with high-risk human papilloma virus (HPV) (3). Majority of HNSCC patients present with locoregionally advanced

(LA-HNSCC) primary disease with concurrent chemoradiation is the standard treatment of care (4). The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor of ErbB family that is overexpression in about 80% of the HNSCC tumors (5). Cetuximab, a monoclonal antibody (mAb) targeted against extracellular domain of EGFR (blocks ligand binding to the receptor thereby inhibiting the downstream signaling) is the only targeted therapy approved for the treatment of LA-HNSCC patients (6). However, addition of cetuximab to chemoradiation regimen, has largely met with limited success in these patients (7). Currently, EGFR targeted therapy is offered indiscriminately to these patients as there are no clinically relevant predictive biomarkers which might be the reason for their limited success. Currently, EGFR targeted therapy is offered indiscriminately to these patients as there are no clinically relevant predictive biomarkers which might be the reason for their limited success (6). Predictive biomarkers of anti-EGFR-based therapy response are well established in colorectal and non-small-cell lung cancer patients (8, 9). However, predictive biomarkers of anti-EGFR based treatment response in HNSCCs are completely lacking. Majority of the studies have analyzed EGFR protein expression, however data are not definitive for its predictive role (10). Although, reports evaluating predictive role of pEGFR dimers and EGFR gene copy number change in LA-HNSCCs are limited in literature (10).

Hypoxia-inducible factor 1α (HIF1α) is a transcription factor regulating numerous cellular processes thereby mediating adaptive responses to hypoxia in cells (11). Meta-analysis have shown that overexpression of HIF1α is significantly associated with poor prognosis in HNSCCs (12). Literature suggests that there is interplay between therapies targeting the epidermal growth factor receptor and hypoxia and HIF1α. Preclinical studies have demonstrated that downregulation of HIF1α expression is to some extent responsible for antitumor activity of

EGFR inhibitors in different cancer including HNSCC (13-15). In addition, HNSCC cells have been shown to be more sensitive to cetuximab under hypoxia in preclinical studies (16, 17). The results reported in these preclinical studies warrant evaluation of HIF1α expression for its predictive value in clinical settings.

Further, cancer stem cells (CSC) are a subpopulation within tumor and have the ability to self-renew, maintain an undifferentiated phenotype and contribute to treatment resistance (18). The cluster of differentiation 44 (CD44), CD98 (SLC3A2) and aldehyde dehydrogenase 1A1 (ALDH1A1) expression are widely studied putative CSC markers in HNSCCs (19-21). Studies have indicated that embryonic stem cells markers SOX2 and OCT4 positive HNSCC cells exhibit CSC like properties (22, 23). Studies have shown poor prognostic association of CSC markers in HNSCC, although their prognostic role is not well established. EGFR pathway is known to be a regulator of CSC population; in addition reports have suggested that CD44 and CD44v6 act as a co receptor for EGFR activation (24). Predictive association of these CSC markers in HNSCC however has not been studied yet.

A recent study by Patil et al. has reported improved progression-free survival (PFS) and loco-regional control (LRC) in unselected LA-HNSCC patients treated with nimotuzumab (humanized IgG1 mAb antibody against EGFR) plus cisplatin radiation compared to the patients treated with only cisplatin radiation in a phase 3 randomized trial conducted in India (25). Targeted therapies are often toxic, marginally effective and costly, in absence of suitable predictive markers these therapies are given to all the patients. Predictive biomarkers of anti-EGFR based treatments response can help in selecting patient group most suitable for thereby improving the benefit to risk ratio. Therefore, in the present study, we have analyzed prognostic

and predictive role of HIF1α expression, EGFR based biomarkers and a panel of CSC markers in LA-HNSCC patients.

**Aim:** To evaluate the prognostic and predictive significance of different biomarkers in HPV-negative LA- HNSCC patients

**Objectives**

- To screen for the presence of HPV infection
- To evaluate EGFR gene copy number change, EGFR and pEGFR protein expression
- To study the expression levels of hypoxia and CSCs markers
- To correlate above results with the treatment outcome and find potential prognostic and predictive biomarkers

**Study design**

Study included participants of a previously published randomized phase 3 trial comparing cisplatin plus radiotherapy with (NCRT) or without nimotuzumab (CRT) in 536 LA-HNSCC patients (25). Treatment-naive formalin-fixed paraffin-embedded (FFPE) tumor biopsy tissues and saliva samples were collected and subjected to HPV screening as per previously published protocol (26). Biopsy tissue with adequate tumor content was available for 432 patients (80%), of which saliva samples were also available for 349 patients. Study was approved by the institutional ethics committee of Tata Memorial Center (IEC approval 50 of 2011). Signed informed consent was obtained from all the participants.

**Results and Discussion**

**Objective 1: To screen for the presence of HPV infection**

**Methodology:** p16 expression was analyzed in 432 patients and DNA-PCR was carried out in 403 samples (either saliva or tissue or both). All the tissue samples with p16 expression in nuclear or cytoplasmic (studied by immunohistochemistry) in ≥10% tumor cells and/or with DNA-PCR positive result in tissue or saliva were subjected to high-risk HPV E6/E7 mRNA in-situ hybridization (RNA-ISH) to confirm the presence of transcriptionally active HPV. A sample was then categorized as HPV-positive on the basis of a positive RNA-ISH result with brown punctate hybridization signals in tumor cell nuclei. p16 status was considered positive if strong to moderate diffuse nuclear and cytoplasmic staining was present in >70% of tumor cells.

**Results:** Overall, 25 (5.8%) cases were positive by RNA-ISH of which 18 (72%) were OPSCC, 2 (8%) were hypopharyngeal SCC and 5 (20%) were laryngeal SCC. In three cases we could not confirm HPV status. Univariate Cox analysis showed that compared to the HPV-negative OPSCC (n=196), HPV-positive OPSCC patients (n=18) had significantly better PFS [HR (95%CI) =0.31 (0.11-0.84)], LRC [HR (95%CI) =0.28 (0.09-0.88)] and OS [HR (95%CI) =0.31 (0.12-0.85)]. Difference in the clinical outcomes was also evident between p16-positive (RNA-ISH negative) and HPV-positive groups, however, the difference reached statistical significance only for OS [HR (95%CI) =0.33 (0.12-0.89)].

**Concluding remark:** India has a low prevalence of transcriptionally active HPV associated HNSCCs. HPV status was strongly associated with better clinical outcomes in OPSCC. p16 IHC is not a good surrogate marker for detecting HPV related HNSCCs.

**Objective 2: To evaluate EGFR gene copy number change, EGFR and pEGFR protein expression and to correlate above results with the treatment outcome and find potential prognostic and predictive biomarkers**

**Methodology**

**Patients:** We excluded 25 HPV positive and additional 3 cases with unconfirmed HPV status and carried out prognostic and predictive analysis in 404 HPV negative patients. Baseline characteristics of the HPV negative patients were balanced between CRT (n=206) and NCRT (n=198) treatment groups and were representative of the total trial population (n=536).

**Biomarker analysis**: Expression of total EGFR and pEGFR dimers-pEGFRY1068 and pEGFRY1173 were analyzed using IHC and EGFR gene copy number was analyzed by Fluorescence in situ hybridization (FISH). IHC staining was evaluated semi-quantitatively by pathologists (blinded to randomization and outcomes) by deriving H-score (0-300) which is a product of percentages of tumors cells showing staining (0-100%) and intensity of staining 1 (weak), 2 (moderate) and 3 (strong). EGFR gene copy status was classified into two categories as FISH-negative (disomy, trisomy and low polysomy) or FISH-positive (high polysomy and/or EGFR gene amplification).

**Statistical analysis:** Median follow-up was 39.13 months. Four-year survival rates are reported. Of 404 study patients, a total of 195 patients (48.3%) had died, 188 (46.5%) progressed and 155 (38.4%) experienced loco-regional failure at the time of analysis. For assessing the prognostic significance of each biomarker (low/negative vs high/positive), only patients from the CRT arm were included in the analysis (27). For assessing the predictive role of biomarker, Cox models were fit, which included treatments (NCRT vs CRT), biomarker status (low/negative vs high/positive) and the interaction between treatment effect and biomarker status (27).

**Results**

**Prognostic role of EGFR based biomarkers:** EGFR expression (membrane or cytoplasmic) studied at different Hscore cutpoints including the median did not associate with PFS, LRC or OS of the patients in the CRT group in univariate Cox analysis. In CRT group, patients with

negative pEGFRY1068 status showed improved PFS compared to patients with positive pEGFRY1068 [HR (95% CI) = 0.63 (0.40–1.0)]. pEGFRY1173 and EGFR–FISH status however, did not show any association with the clinical outcomes in CRT group.

**Predictive role of EGFR based biomarkers:** Patients with high-membrane EGFR categorized at median cutpoint, univariate Cox analysis showed that PFS [HR (95% CI) = 0.61 (0.41–0.92)] and LRC [HR (95% CI) = 0.59 (0.38–0.92)] were significantly improved with NCRT versus CRT. Similar improvements were not observed in the patients with low membrane EGFR expression. Statistically significant better PFS [HR (95% CI) = 0.58 (0.37–0.90)] and LRC [HR (95% CI) = 0.51 (0.31–0.85)] but not OS [HR (95% CI) = 0.76 (0.49–1.18)] were also observed in the patients with high cytoplasmic EGFR expression (categorized at median cutpoint) with NCRT versus CRT. Similar benefits were not observed in the patients with low cytoplasmic EGFR. Statistically significant interaction between treatment effect and EGFR (membrane or cytoplasmic) expression status was not observed for any of the studied endpoints. However, NCRT improved the outcomes in patients with negative pEGFRY1068 status [PFS: HR (95% CI) = 0.66 (0.48–0.92); LRC: HR (95% CI) = 0.63 (0.44–0.90); OS: HR (95% CI) = 0.71 (0.52–0.96)], but offered no benefit in patients expressing pEGFRY1068 dimer. Further, we observed longer LRC in patients with negative pEGFRY1173 with NCRT versus CRT [HR (95% CI) = 0.66 (0.45–0.97)]; however, significant improvements in PFS were observed in patients with positive pEGFRY1173 [HR (95% CI) = 0.52 (0.29–0.94)]. We did not find any statistically significant interaction between treatment effect and pEGFR status. In patients with EGFR–FISH negative status NCRT improved PFS [HR (95% CI) = 0.60 (0.40–0.91)] and OS [HR (95% CI) = 0.68 (0.46–0.99)] compared to CRT. Similar improvements were not observed in patients with

EGFR-FISH positive status. EGFR-FISH and treatment effect did not show any significant interaction.

**Concluding remark:** Patients with tumors expressing pEGFR dimers were small in number as majority of the HNSCCs were negative for pEGFR expression, therefore, these results should be interpreted carefully and needs to be further validated. Overall, the treatment effect of NCRT is independent of expression of EGFR and EGFR gene copy status and therefore additional biomarkers needs to be analyzed.

**Objective 3: To study the expression levels of hypoxia and CSCs markers and to correlate above results with the treatment outcome and find potential prognostic and predictive biomarkers**

**Methodology:** Same as that of objective 2 (page no.6-7). Briefly, 404 HPV-negative patients were analyzed for HIF1α and CSC marker expression blinded to treatment allocation and patient's outcomes. Expression of biomarkers was studied using IHC. IHC staining was evaluated semi-quantitatively by deriving H-score.

**Results:**

**Prognostic role of HIF1α and CSC markers:** Low HIF1α expression was associated with better PFS, LRC and OS in the CRT group. Analyses using the median cutpoint showed that the low HIF1α expression was significantly associated with better LRC [HR (95% CI) = 0.58 (0.38–0.89)] as well as OS [HR (95% CI) = 0.62 (0.42–0.91)], and showed a trend towards improved PFS [HR (95% CI) = 0.69 (0.47–1.01)]. Multivariable analysis adjusted for confounding variables (age, clinical stage and site of tumor) identified HIF1α as an independent negative prognostic biomarker for PFS, LRC and OS. Further validation by bootstrap resampling method

confirmed the prognostic effect of HIF1α. Of the five CSC markers, only CD98 expression showed negative prognostic association. Low expression of CD98 was associated with longer OS [HR (95%CI)= 0.63 (0.41-0.96)] and LRC [HR (95%CI)= 0.66 (0.41-1.04) in CRT group at the cutpoint of 40. Multivariable analysis confirmed independent prognostic significance of CD98 expression for LRC (P=0.049) and OS (P=0.028). We did not observe any prognostic impact of CD44, CD44v6, ALDH1A1 or SOX2 expression.

**Predictive association of HIF1α expression:** Interestingly, patients with high HIF1α (categorized at median cutpoint) had significantly improved PFS [HR (95% CI) = 0.55(0.37–0.82)], LRC [HR (95% CI) = 0.55 (0.36–0.85)] and OS [HR (95% CI) = 0.54 (0.36–0.81)] with NCRT compared to CRT. Similar improvements were not observed in low HIF1α-expressing subgroups. A statistically significant qualitative interaction was observed between treatment effect and HIF1α status for OS [P = 0.008] but not for PFS [P=0.137] or LRC [P=0.234]. The predictive role of HIF1α was further validated internally by bootstrap resampling method. Analysis carried out at different possible Hscore cutpoints revealed that overall high HIF1α expression was numerically associated with better outcomes in NCRT as compared to CRT.

**Predictive association of different CSC markers:** HRs of progression, loco-regional failure and death were lower regardless of the cutpoint used to define low-CD44 expression. Test for interactions between CD44 status and treatment was significant only for OS at the cutpoints of 140 (*P*=0.022) and 150 (*P*=0.009). Similar trend was also observed with CD44v6 expression. HRs of progression, loco-regional failure and death were generally lower regardless of the cutpoint used to define low-CD44v6 expression. At cutpoint of 40, the interaction P value was significant for LRC (P=0.023) and OS (P=0.036) but not for PFS (P=0.075). Overall, HRs of progression, loco-regional failure and death were lower regardless of the cutpoint used to define

low or high CD98 expression. No statistically significant interaction was obtained at any of the studied cutpoints. HRs of progression, loco-regional failure and death were generally significantly lower regardless of the cutpoint used to define low-ALDH1A1 expression. We did not find a statistically significant interaction *P* value at any of the studied cutpoints for any of the clinical end point. Overall, HRs of progression, loco-regional failure and death were significantly lower at most of the cutpoint used to define low or high SOX2 expression. We did not observe any significant interaction between SOX2 status and treatment effect. We did not observe nuclear OCT4A staining in any of the tumor, similar results are also reported recently by Mallo et al in HNSCC (28).

**Concluding remark**: Nuclear HIF1α and complete membrane CD98 expression are independent negative prognostic factors in HPV negative HNSCC. Quantitative interaction was observed between treatment effect and HIF1α status for OS. Addition of nimotuzumab to CRT improved outcomes in patients with high HIF1α. In addition, complete membrane expression of CD44 and CD44v6 might be helpful in predicting the treatment response to NCRT.

**Summary and major conclusions derived from the study:**

For LA-HNSCC patients, the identification of predictive biomarkers to enable selection of patients for a specific treatment is a pressing need. In the present study, we analyzed prognostic and predictive impact of different biomarkers in HPV negative HNSCC patients in randomized setting. We showed that HIF1α expression was predictive of CRT treatment response. For patients with high HIF1α expression, addition of nimotuzumab to CRT might improve clinical outcomes compared to patients receiving only CRT. CD44 and CD44v6 might help in predicting NCRT treatment response in HPV negative HNSCC patient. As this was a single center study, results need further independent validation in a multicentric cohort.

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**a. Published**

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- Prognostic and predictive significance of nuclear HIF1α expression in locally advanced HNSCC patients treated with chemoradiation with or without nimotuzumab. Patel U, Pandey M, Kannan S, Samant TA, Gera P, Mittal N, Rane S, Patil A, Noronha V, Joshi A, Patil VM, Prabhash K, Mahimkar MB. Br J Cancer. 2020 Dec; 123(12):1757-1766. doi: 10.1038/s41416-020-01064-4. PMID: 32939054.
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**b. Communicated**

Prognostic and predictive roles of cancer stem cell markers in head and neck squamous cell carcinoma patients receiving chemoradiotherapy with or without nimotuzumab. Usha Patel, Sadhana Kannan, Swapnil U Rane, Neha Mittal, Poonam Gera, Asawari Patil, Subhakankha Manna, Vishwayani Shejwal, Vanita Noronha, Amit Joshi, Vijay M Patil, Kumar Prabhash, Manoj B Mahimkar.

**Other Publications:**

**a. Book/Book Chapter**

None

**b. Conference**

- Poster presentation entitled "Study of correlation between EGFR, hypoxia markers and cancer stem cell markers in locally advanced HNSCC" at 37th IACR Convention, held in Kolkata, India on February 23-25, 2018.
- Poster presentation entitled "Nuclear HIF1α a predictor of survival for nimotuzumab along with CTRT treated HPV negative advanced HNSCC patients" at 4th EACR

Conference on Cancer Genomics, held in Cambridge, United Kingdom on 23-26 June, 2019.

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Date: 18/12/2020

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**TABLE OF CONTENT**

## LIST OF FIGURES

## LIST OF TABLES

# 1.

# Introduction

# and

# Review of literature

## 1.1. Head and neck squamous cell carcinomas

Head and neck cancers (HNC) are a heterogeneous group of cancers arising in the epithelial cells of the mucosal linings of the upper aero-digestive region of head and neck. Histologically more than 90% of head and neck cancers are squamous cell carcinoma (HNSCCs). Anatomically HNSCCs are majorly divided into cancers of the oral cavity, oropharynx, hypopharynx, and larynx (Figure 1), which can be further subcategorized into 14 subsites according to the International Classification of Diseases 10$^{th}$ revision (ICD-10).

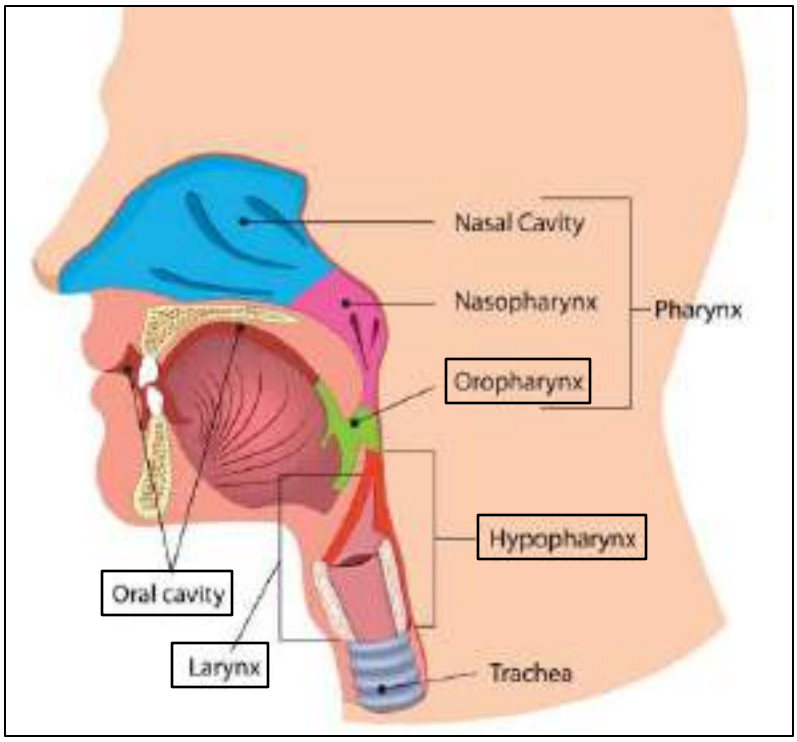



***Figure 1: Diagram showing the major anatomical sites in the head and neck region.*** *Squamous cell carcinomas of the oral cavity, oropharynx, larynx, and hypopharynx are commonly grouped as HNSCCs as they usually share common etiologic factors (Source: https://www.nfcr.org/blog/head-and-neck-cancer-awareness-month/)*

### 1.1.1. Epidemiology of HNSCCs

HNSCCs remain a significant cause of morbidity and mortality and comprise a major cancer burden in many economically developing countries including India (1, 2). The actual cancer incidence and mortality in India is higher than the estimated as many cases of HNSCCs go undiagnosed or unreported (1). Estimated new cases reported in 2018 in Indian males were 92,011 cases of lip and oral cavity cancers (age-standardized rate (ASR)= 3.9); 25,834 cases of larynx SCC (ASR=4.2); 20,668 cases of hypopharynx SCC (ASR= 3.3) and 15,529 cases of OPSCC (ASR= 2.5) according to GLOBOCAN (IARC). HNSCCs are more prevalent in males as compared to females. In males, SCC of the lip and oral cavity are prevalent followed by the larynx, hypopharynx, and oropharynx (Figure 2).

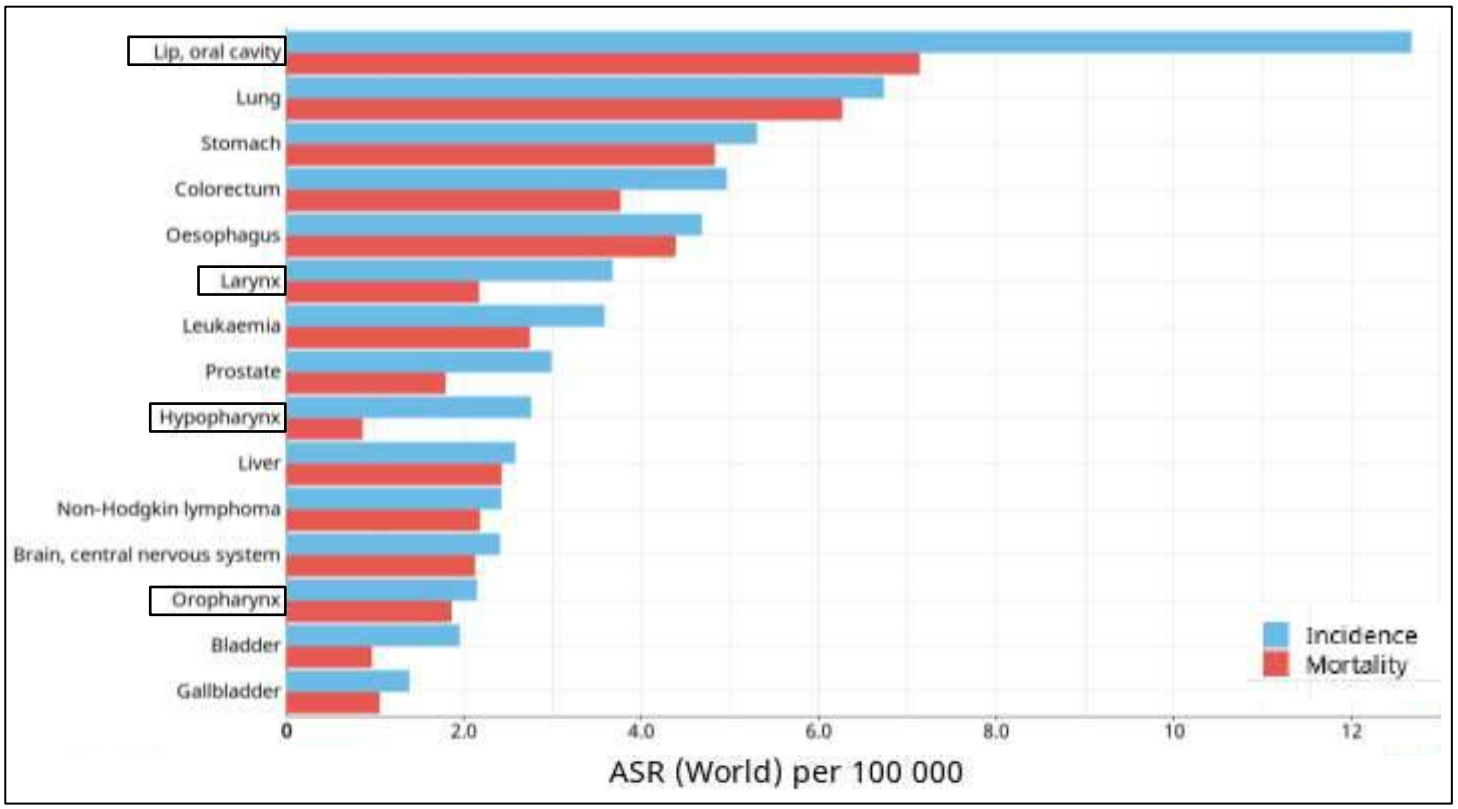



***Figure 2: Bar chart showing incidence and mortality of HNSCCs according to GLOBOCAN 2018 estimates.*** *Age-standardized incidence and mortality rates of top the 15 cancers in Indian males*

Similar estimates for Indian females are 27,981 cases of lip and oral cavity cancers (ASR= 4.3); 5,279 cases of hypopharynx SCC (ASR=0.81); 2,887 cases of larynx SCC (ASR=0.45) and 2,374 cases of oropharyngeal SCC (OPSCC) (ASR= 0.37).

### 1.1.2. Etiology of HNSCCs

**1.1.2.1. Tobacco and alcohol:** Tobacco use and alcohol consumption are the classical risk factors for the development of HNSCC hence HNSCCs are largely a preventable disease (29). Major classes of carcinogens present in tobacco and tobacco smoke are tobacco-specific nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, aldehydes, and certain volatile organics (30). These carcinogens undergo metabolic activation mediated by cytochrome P450 enzymes and get converted into ultimate carcinogens which are able to directly attack and form covalent adducts with DNA bases (30). Alcohol is oxidized by alcohol dehydrogenases and/or by oral microflora to acetaldehyde which is able to react with DNA and form DNA adducts (31). These risk factors are generally similar for the oral cavity, pharynx, and larynx, although the magnitude of risk may vary (29). In India, different tobacco habits including various forms of smokeless (eg. betel quid, gutka, pan masala, khaini, mawa, mishri) or smoked tobacco (eg. bidi, cigarettes, cigars, hookah) products are used singly or in combination (32, 33).

**1.1.2.2. Human papilloma virus:** Human papilloma viruses (HPV) are small non-enveloped double-stranded DNA viruses. The tumorigenic potential of HPV is majorly determined by the activity of two early genes E6 and E7 (Figure 3) (34) Protein products of two early viral genes E6 and E7 inhibit and degrade two important cellular tumor suppressors- p53 and the retinoblastoma protein (pRB) respectively (35). On the basis of the oncogenic potential

of different HPV types, they are classified as high risk or low risk. HPV infects epithelial cells and depends on the differentiation pathway of epithelial cells to complete its lifecycle. Infection with high-risk HPV is a major etiological factor for 70-80% of cervical cancers (36). HPV is now established as a novel independent risk factor for HNSCC having an anatomical preference for the base of tongue and tonsils subsites of the oropharynx (37). More than 90% of OPSCC are caused by high-risk HPV type 16.

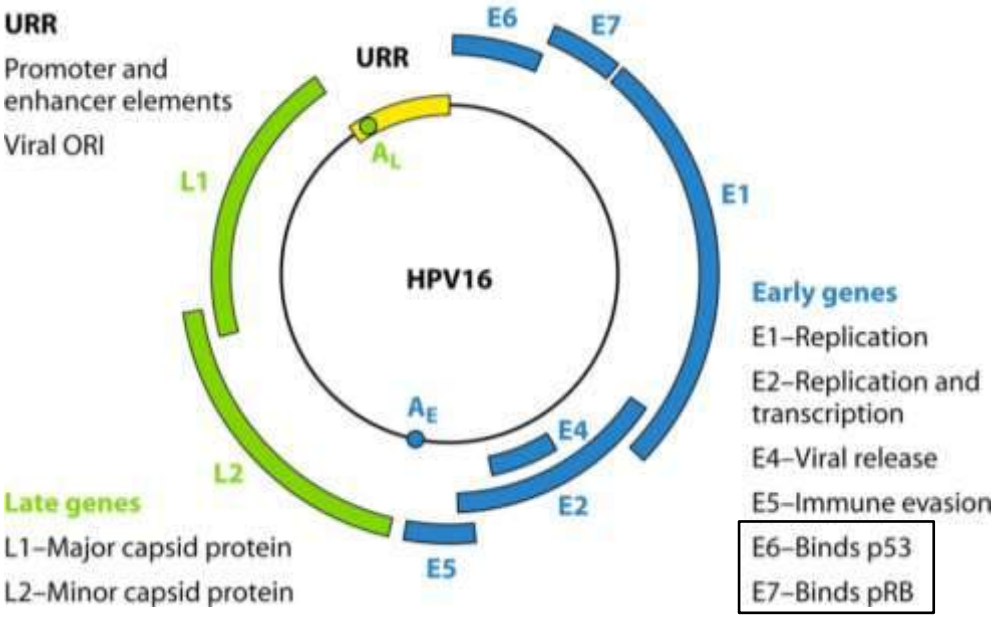



***Figure 3: Diagram showing the genomic organization of human papillomavirus (HPV) type 16.***

*HPV is a double-stranded DNA virus of about 8 kb that can be divided into three regions as determined by their functions; upper regulatory region (URR), and two coding regions, early (E) and late (L) coding regions. The early region encodes for regulatory proteins E1, E2, E4-7 necessary for viral replication and the late region encodes for the structural proteins L1-L2 involved in virion assembly (38)*

The molecular profiles, including mutations, copy number alterations, and gene expression patterns of HPV positive tumors are reported to be distinct from those of HPV negative tumors (3, 39). Epidemiological evidence has shown that HPV prevalence in HNSCCs varies substantially among different anatomical sites and geographic regions (40). HPV related OPSCC cases are rising in economically developed countries among males, as well as females (40). The prevalence of HPV related HNSCC in India ranges widely across the studies due to differences in the methods of HPV detection, demographic groups, data reporting, and a lack of clear definition of HPV positivity as summarized by Bhosale et al and Nair et al (26, 41).

**1.1.2.3. Other risk factors**: Additional risk factors for HNSCC includes host genetic factors including polymorphism in genes coding carcinogen metabolizing enzymes and DNA repair (42-44), exposure to UV radiation or industrial carcinogens, chronic irritations by a sharp tooth or a loose denture which is associated with risk of oral cancers and poor oral hygiene.

### 1.1.3. HNSCC Staging

Staging of HNSCCs are done according to The American Joint Committee on Cancer's (AJCC) and Union for International Cancer Control (UICC) TNM classification which is based on three key components: **T** (**T**umor size); **N** (Lymph **N**ode metastasis) and **M** (Distant **M**etastasis)

The new (eighth) edition of AJCC staging was published in 2016 and became effective in January 2018. It has now a separate TNM staging system for HPV-associated (p16 positive) OPSCC given their significantly favorable outcomes as compared to HPV negative OPSCC (Table 1-2). p16 overexpression is a surrogate marker of HPV infection and p16 immunohistochemistry (IHC) is now incorporated in the clinical practice as a risk stratification

marker for OPSCC patients as stated above. Although, recent evidence suggest that p16 IHC lacks the specificity for detecting transcriptionally active HPV and detection of oncogenes E6/E7 mRNA is now regarded as the gold standard (26, 45-48).

***Table 1: Staging of oral cavity, laryngeal, hypopharyngeal and p16 negative oropharyngeal cancers (AJCC, 8th Edition)***

| | | | | |
|---|---|---|---|---|
| | Stage 0 | Tis | N0 | M0 |
| Early stage | Stage I | T1 | N0 | M0 |
| | Stage II | T2 | N0 | M0 |
| Advanced stage | Stage III | T3 | N0 | M0 |
| | | T1,T2,T3 | N1 | M0 |
| | Stage IVA | T4a | N0, N1 | M0 |
| | | T1,T2,T3,T4a | N2 | M0 |
| | Stage IVB | Any T | N3 | M0 |
| | | T4b | Any N | M0 |
| | Stage IVC | Any T | Any N | M1 |

***Table 2: Staging of p16 positive oropharyngeal cancers (AJCC, 8th Edition)***

| | | | | |
|---|---|---|---|---|
| Early stage | Stage I | T1,T2 | N0,N1 | M0 |
| | Stage II | T1,T2 | N2 | M0 |
| | | T3 | N0,N1,N2 | M0 |
| Advanced stage | Stage III | T1,T2,T3,T4 | N3 | M0 |
| | | T4 | N0,N1,N2,N3 | M0 |
| | Stage IV | Any T | Any N | M1 |

## 1.2. Treatment and prognosis of HNSCC patients

The major treatment modalities for HNSCC patients include surgical resection, radiotherapy, and chemotherapy. All modalities, however, severely reduce the quality of life. Treatment can be of single modality or multi-modality. Treatment decision depends on different variables among which the major factors are tumor site, disease stage (TNM stage), age, and performance status of the patient.

**1.2.1. Early stage cancers**: About one-third of HNSCC patients present with early-stage cancer. These patients are treated with surgery or radiotherapy and generally have a better prognosis.

**1.2.2. Advanced stage cancers**: Almost two-thirds of patients present with loco-regionally advanced disease (LA-HNSCC) and have a poor prognosis. For patients with resectable tumor surgery with adjuvant radiotherapy or adjuvant radio-chemotherapy is offered. For unresectable tumors and organ preservation approach, concurrent chemoradiation is the standard treatment of care.

## 1.3. Prognostic and predictive biomarkers

Prognostic and predictive biomarkers can aid in the rational development of anticancer drugs. Prognostic biomarkers give information for patient risk stratification and predictive biomarkers provide information for the choice of the treatment for a patient or a group of patients. In literature, these two terms are not clearly defined and are used interchangeably.

***1.3.1.*** **Prognostic biomarkers:** Prognostic biomarkers are used to predict the natural course of a tumor. Prognostic biomarkers can indicate whether the outcome for the patient is likely to be good or poor irrespective of the treatment. Thus they can guide the decision of whom to treat (or

how aggressively to treat). Prognostic biomarkers are also important in designing clinical trials for stratifying patients into homogenous subgroups. A control group from a randomized trial is an ideal setting for evaluating the prognostic value of a biomarker (27). Prognostic biomarkers can show a positive or negative association. If the presence of biomarker is associated with better clinical outcomes then biomarker is a positive prognostic factor and if the presence of biomarkers is associated with poor clinical outcomes then biomarker is a negative prognostic factor. An example of a well-established positive prognostic biomarker or risk stratification factor in HNSCC is HPV status which is associated with favorable clinical outcomes irrespective of the treatment (49, 50). Prognostic role of HPV in OPSCC is well established in Western and European countries; however, such studies from India are far limited (51). The sensitivity of HPV-positive HNSCC to radiotherapy and chemotherapy is higher than that of HPV-negative HNSCC (52, 53). Hence, patients with HPV-positive HNSCC are associated with enhanced survival. The molecular mechanisms involved in HPV-associated enhanced chemo-radio sensitivity are not very clear (54). HPV-positive tumors may harbor a decreased number of CSCs leading to improved treatment outcomes of these patients. However, there are conflicting reports on the correlation between the CSC population and HPV status (55-60). Additionally, there are limited studies that have analyzed the expression of different putative CSC markers in the clinical samples.

**1.3.2. Predictive biomarkers:** Unlike prognostic biomarker, a predictive biomarker is associated with response or lack of response to a specific treatment. In other words a biomarker is predictive if the relative efficacy of two treatments is different for biomarker positive patients compared with biomarker negative patients. Ideally predictive significance of biomarkers is estimated in a randomized setting comparing experimental intervention with standard treatment

of care in patients with and without the biomarker. Distinguishing prognostic biomarkers and predictive biomarkers can be difficult as they are not always exclusive and some biomarkers are both prognostic and predictive. To determine whether a biomarker is potentially predictive, a formal test for an interaction between the biomarker and treatment group is required; a statistically significant interaction will indicate that the treatment effect differs by biomarker status (61). A predictive biomarker can show a qualitative or quantitative effect (61, 62). A qualitative effect is when one biomarker subgroup benefits from treatment and the other obtains no benefit (or is harmed) from treatment. If both subgroups benefit from the treatment, it is a quantitative interaction. In that case, one subgroup will derive much more benefit compared to another subgroup. Unlike quantitative interactions, qualitative interactions can provide a clear indication of treatment choice (62).

Targeted therapies are often marginally effective, toxic, and costly. Lack of suitable predictive biomarkers results in many patients receiving a treatment from which they do not benefit but can experience toxicity. Suitable predictive biomarkers not only help in improving the benefit to risk ratio but also gives insights to understand the mechanisms of sensitivity and resistance to a specific treatment and in the development of improved therapeutic strategies to overcome the resistance. Therefore, there is a great need to identify the biomarkers which are predictive of response to these targeted therapies which can help in selecting the patient group most suitable for a particular therapy thereby improving the benefit to risk ratio and decrease medical costs.

### 1.3.3. Limitation of predictive biomarker studies

There is a lack of clarity between the prognostic and predictive association of biomarkers. The best setting for evaluating a predictive biomarker for targeted therapy is a randomized clinical

trial of the targeted therapy versus standard therapy. However, many studies have demonstrated the treatment benefits associated with biomarker status in patients treated uniformly with the targeted therapy. This is insufficient to demonstrate predictive significance as a biomarker may solely be prognostic in this case. In addition, all predictive biomarker studies do not perform the statistical test for treatment-by-biomarker interaction which is needed to ensure that the observed treatment effect is not a result of chance or random event.

### 1.4. EGFR targeted therapy in HNSCCs

The epidermal growth factor receptor (EGFR) is a cell membrane tyrosine kinase receptor of the ErbB family, playing important role in cell proliferation, angiogenesis and metastasis through complex downstream signaling pathways such as PI3K/Akt and MAPK pathways. Deregulation of the EGFR signaling pathway is frequently observed in many epithelial cancers including HNSCCs. EGFR overexpression and its gene locus (7p11.2) amplification are observed in about 80% and 10-30% of HNSCC tumors respectively and are associated with poor clinical outcomes (5, 63-65). EGFR gene mutations however are rarely observed in HNSCC tumors (66). Different EGFR inhibitors are approved for the treatment of different epithelial cancers. EGFR inhibitors are classified into two classes- monoclonal antibody (mAb) against the extracellular domain of EGFR (eg. cetuximab, panitumumab, nimotuzumab), which inhibits ligand binding to the receptor and small molecule tyrosine kinase inhibitors (TKIs) like erlotinib or gefitinib, which binds at ATP binding site in the kinase domain of EGFR. The overall response rate to cetuximab as a single agent treatment approach analyzed in recurrent/metastatic HNSCC does not exceed 13% (67). For locally advanced HNSCCs EGFR mAbs are given in combination with radiotherapy or radio-chemotherapy. Cetuximab, a chimeric IgG1 therapy is the only targeted

therapy approved by the FDA (Food and Drug Administration) for the treatment of LA-HNSCCs (68, 69). The addition of cetuximab to the chemoradiation regimen has largely met with limited success in these patients (7). Nimotuzumab (h-R3), a humanized IgG1 mAb has demonstrated a unique clinical profile, wherein anti-tumor activity was observed in absence of severe skin, renal, gastrointestinal mucosa toxicities commonly associated with EGFR targeting antibodies, cetuximab and panitumumab (70, 71). A recent study by Patil et al have reported improved progression-free survival (PFS) and loco-regional control (LRC) in unselected LA-HNSCC (more than 94% were HPV negative) patients treated with nimotuzumab along with cisplatin-radiation compared to the patients treated with only cisplatin-radiation in a large Phase 3-randomized trial which was conducted at Tata Memorial Center, Mumbai, India (25).

### 1.5. Predictive biomarkers of EGFR targeted therapy response

Clinically used predictive biomarkers for anti-EGFR based treatment include activating mutations in the EGFR kinase domain that are associated with sensitivity to gefitinib and erlotinib in patients with advanced non-small cell lung cancers (NSCLC) (72, 73). Another important predictive biomarker is activating mutations in the *KRAS* gene which are associated with a lack of sensitivity to EGFR mAbs like cetuximab in patients with metastatic colorectal cancers (mCRC) (8, 74). These predictive biomarkers of anti-EGFR treatment are approved for clinical use and have substantially improved the clinical management of patients with advanced NSCLC or mCRC. However, mutations in EGFR (4%) or RAS gene (*HRAS*: 4%; *KRAS*: 0.4%; *NRAS*: 0.4%) are comparatively rare in HNSCCs and therefore they have limited clinical utility (66). Currently, EGFR targeted therapy is given indiscriminately to the LA-HNSCC patients as there are no clinically relevant predictive biomarkers.

## 1.6. Candidate predictive biomarkers of EGFR targeted therapy response in HNSCC

### 1.6.1. Epidermal growth factor receptor

EGFR protein expression is the most commonly studied biomarker for its predictive role in anti-EGFR treatment response. However, the majority of the studies have reported a limited predictive role of EGFR protein expression in HNSCC patients as reviewed by Bossi et al (10). Studies evaluating the predictive value of EGFR gene amplification and expression of phospho EGFR (pEGFR) dimes are however far limited in the literature (10).

### 1.6.2. Hypoxia inducible factor 1α

The hypoxic microenvironment is a common feature associated with chemo-radio resistance in solid tumors including HNSCC (75). Upon hypoxia, as an adaptive response cell increases the activity of hypoxia inducible factor 1α (HIF1α), which is a transcription factor and major effector molecule of hypoxia, HIF1α promotes transcription of many key genes involved in angiogenesis, cell survival, glucose metabolism, and invasion (76). In addition to hypoxia, aberrant growth factor signaling including that of EGFR is shown to upregulate the activity of HIF1α (77, 78). Interestingly, preclinical studies have repeatedly demonstrated that inhibition of EGFR signaling leads to downregulation of HIF1α and its transcription targets, suggesting that anti-tumor effects of EGFR targeted therapies to some extent might be dependent on downregulation of HIF1α (13-15, 79-81). Also, studies have shown that HNSCC cell lines are more sensitive towards cetuximab under hypoxic conditions as compared to normoxic conditions (17, 82). These preclinical studies warrant clinical evaluation of the predictive impact of HIF1α.

HIF1α expression has been studied widely for its prognostic role; however its predictive impact for anti-EGFR treatment response has not been evaluated in HNSCC patients.

### 1.7. Prognostic and predictive role of putative cancer stem cell markers in HNSCCs

Cancer stem cells (CSC) are a subpopulation within a tumor with the ability to self-renew, maintain an undifferentiated state, contribute to therapy resistance, and repopulate tumor heterogeneity upon recurrence or metastasis (18). CSCs can be distinguished using the differential expression of protein markers (83). The cluster of differentiation 44 (CD44), CD98, and aldehyde dehydrogenase 1A1 (ALDH1A1) expression are widely studied putative CSC markers in HNSCCs (19-21).

CD44 is a cell adhesion molecule of class I transmembrane glycoprotein family mediating cell-cell and cell-matrix interactions thereby playing roles in growth, survival, differentiation, and motility of cells (84). CD44 is the major receptor of hyaluronic acid (HA) (85). CD44 function is regulated by glycosylation as well as by alternative splicing CD44 gene consist of 20 exons out of which 10 can be regulated by alternative splicing (called as variable exons) giving rise to different isoforms (84). The smallest isoform (CD44) does not contain any variable exons and is expressed on most vertebrate cells including immune cells. However, expression of other splice variants (CD44v1-v10) is tissue-specific and is shown to be overexpressed in different cancers including HNSCC (86). CD44 and its isoforms are shown to be involved in the progression and metastasis of different cancers (87). Interestingly, It has been shown that activated CD44 upon HA binding interacts with different tyrosine kinase receptors and activates downstream Rho-GTPase and Ras-MAPK signaling pathways (88). Reports have shown that CD44 and its isoforms interact with ErbB family members in breast cancer, NSCLC, and HNSCC and can act as a co-receptor for activation of ErbB family receptors (24, 89, 90). In HNSCC, CD44 and its

variants are largely studied for their prognostic role (86, 91) . However, studies evaluating the predictive role of CD44 and its variants are far limited in the literature.

CD98 heavy chain (CD98hc, 4F2hc, SLC3A2) is a type II single-pass transmembrane glycoprotein. CD98hc is expressed on proliferating lymphocytes and other proliferating cells. CD98hc is shown to play a role in β-integrin signaling which is involved in cell spreading and tumorigenesis (92). CD98hc also interacts with LAT1 (multi-pass light chain of large neutral amino acid transporters) through a disulfide bond and acts as a chaperone by promoting LAT1 trafficking, functional insertion, and stabilization into the plasma membrane (93). Digomann et al have recently shown that the high expression levels of CD98hc lead to increased mTOR pathway activation, amino acid metabolism and DNA repair; and downregulation of oxidative stress and autophagy which leads to radiation resistance and poor prognosis in HNSCCs (94).

ALDH1A1 belongs to the superfamily of NADP(+)-dependent enzymes which catalyze the oxidation of aliphatic and aromatic aldehydes (95). ALDH1A1 also oxidize retinal to retinoic acid, retinoic acid bind to its nuclear receptor and drives transcription of genes involved in growth, differentiation, and maintenance of adult tissues and organs. High expression of ALDH1A1 is reported to be a negative prognostic factor in many cancers including HNSCCs (96).

SOX2 (Sex-determining region-Y homeobox-2) and OCT4 (Octamer-binding transcription factor 4, also known as POU5F1) are important pluripotency-associated transcription factors involved in the maintenance of self-renewal capacity of embryonic stem cells (97, 98). Overexpression of SOX2 and OCT4 has been studied for their prognostic role in HNSCC patients, however, contrasting reports exist (99-101). Studies have indicated that SOX2 and OCT4 positive HNSCC cells exhibit CSC-like properties (22, 23).

To improve the efficacy of a given treatment, a better knowledge of molecular profiles and their impact on treatment outcomes is required. Therefore, we have assessed the prognostic and predictive role of EGFR based biomarkers, HIF1α, and a panel of CSC markers in a series of patients with HPV negative LA-HNSCC treated with cisplatin-radiation with or without nimotuzumab in a randomized setting.

# 2. Aims and Objectives

## 2.1. Aim of the study

To evaluate the prognostic and predictive significance of different biomarkers in HPV negative locally advanced stage HNSCC patients.

## 2.2. Objectives of the study

- To screen for the presence of HPV infection
- To evaluate EGFR gene copy number change, EGFR and pEGFR protein expression
- To study the expression levels of hypoxia and CSCs markers
- To correlate above results with the treatment outcome and find potential prognostic and predictive biomarkers

# 3.

# Materials and Methods

## 3.1. Study design

### 3.1.1. Patients

The study included participants of a randomized phase-3 clinical trial (CTRI/2014/09/004980) conducted between 2012 and 2018 at Tata Memorial Center (TMC), Mumbai, India (25). Briefly, 536 LA-HNSCC patients with newly diagnosed, treatment-naive, non-metastatic, stage III or IV LA-HNSCC arising in the oropharynx, larynx, hypopharynx, or oral cavity were blindly randomized 1:1 to receive radical radiotherapy (66-70 Grays) with concurrent weekly cisplatin (30 mg/$m^2$) (CRT arm) or the same schedule of cisplatin-radiation with weekly nimotuzumab (200 mg) (NCRT arm). The present biomarker study was approved by the institutional ethics committee of Tata Memorial Center (IEC approval 50 of 2011). A separate informed consent was obtained from all the patients (Sample of informed consent form is provided in the appendix section).

### 3.1.2. Sample collection

Treatment naïve formalin-fixed paraffin-embedded (FFPE) tumor biopsy tissues and saliva samples were collected. Five µm thick section of each FFPE tumor tissue was stained with Hematoxylin and Eosin and was then evaluated by a pathologist to confirm the presence of adequate tumor with SCC histology. Out of 536 patients, FFPE biopsy tissue with adequate tumor content was available for 432 patients (80%) and saliva sample was available for 433 patients which were subjected to HPV screening using a previously published algorithm (26). The overall workflow of the study is outlined in Figure 4.

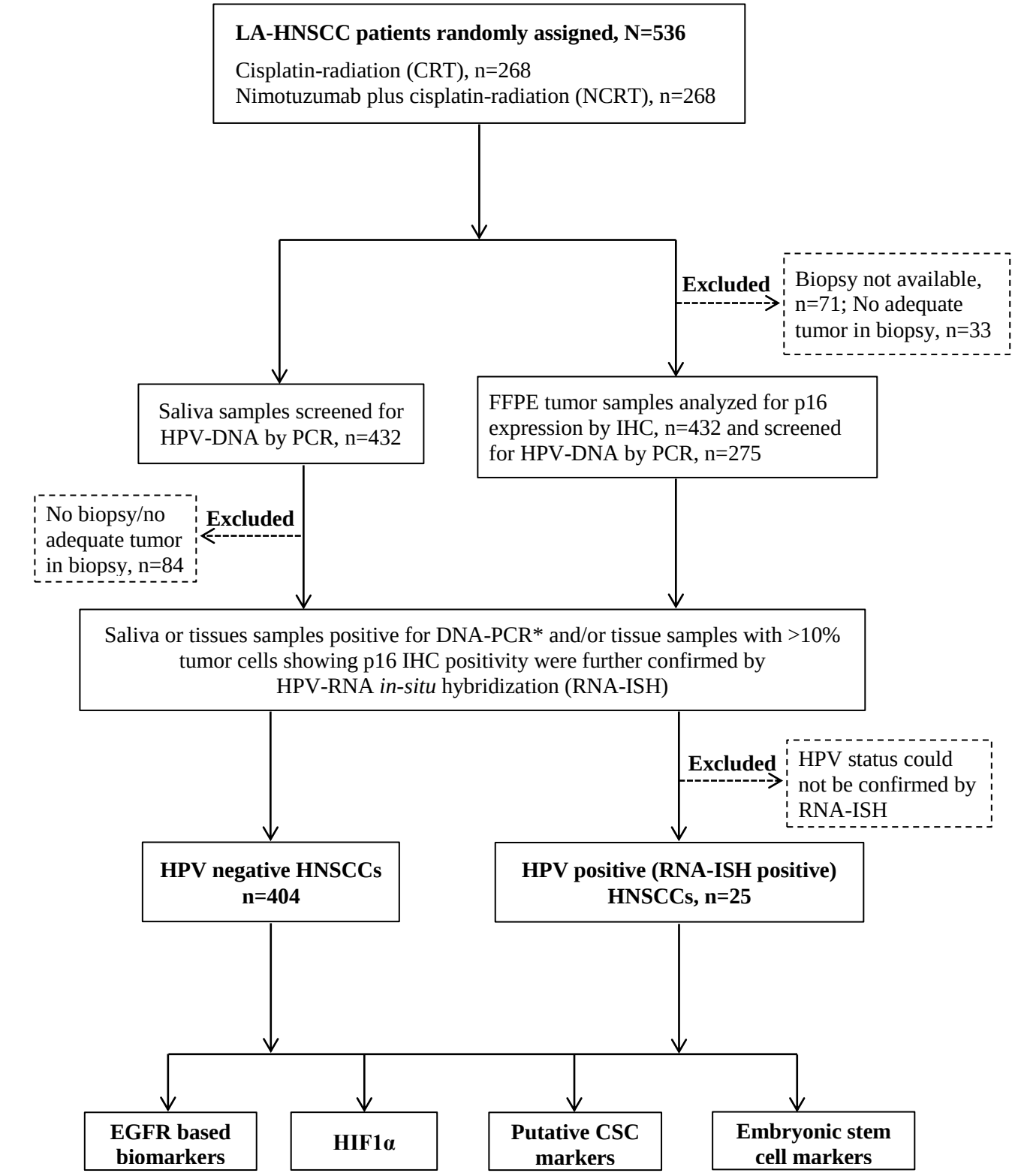



***Figure 4: The overall workflow of the study***

### 3.2. DNA extraction from tumor tissues and saliva samples

Genomic DNA was extracted from FFPE tissues by column purification using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 10 µm thick freshly cut sections were deparaffinized with xylene, washed with 100% ethanol, and digested using proteinase K at 55°C overnight, followed by binding of DNA to the column (QIAamp DNA FFPE Tissue Kit, Qiagen, Hilden, Germany), washing and elution. Sectioning and DNA extractions were carried out with the highest measures to avoid cross-contamination. Genomic DNA from saliva was extracted according to the manufacturer's instructions (DNA Genotek Inc., Ontario, Canada). Briefly, saliva samples were heated at 56°C for 6 h. 500uL of saliva was treated with a purifier (OG-L2P) to obtain clear supernatant which was subjected to absolute ethanol treatment for DNA precipitation, after washing step with 70% ethanol, DNA pellet was dissolved in autoclaved MilliQ water.

### 3.3. Nested PCR

Genomic DNA extracted from FFPE tissue and saliva was subjected to nested PCR using MY09/MY11 primers. PCR product of this PCR (450bp) was amplified using GP5+/GP6+ primers by touch down PCR. Diluted DNA (0.05ng/µL) from the HeLa cell line (Cervical cancer cell line, HPV18 positive) was used as a positive control for MY PCR. For GP+ PCR, 1.100 diluted MY product was used as positive control. Care was taken to avoid cross-contamination between the samples and the positive control. Nested PCR products were sequenced to confirm the HPV type. As a confirmation of amplifiability of DNA, β-globin gene PCR was run on all the DNA samples using PC03/PC04 primers. Details of PCR master mix and conditions are provided in (Table 3-4). FFPE tumor tissue of PCR positive cases (either tissue or saliva) were tested for transcriptionally active HPV by RNA-ISH. Primers used for PCR amplification are listed in

Table 5. All the PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and visualized under UV light.

***Table 3: PCR master mix***

| **Beta-Globin** | | **MY09/MY11** | | **GP5+/GP6+** | |
|---|---|---|---|---|---|
| Components (Stock conc.) | Volume | Components (Stock conc.) | Volume | Components (Stock conc.) | Volume |
| 5XPCR buffer | 5.00 µl | 5XPCR buffer | 5.00 µl | 5XPCR buffer | 2.50 µl |
| 10mM dNTP | 2.50 µl | 5mM dNTP | 1.00 µl | 10mM dNTP | 1.25 µl |
| 25mM $MgCl_2$ | 1.00 µl | 25mM $MgCl_2$ | 4.00 µl | 25mM $MgCl_2$ | 1.75 µl |
| PC03 (10pM) | 1.00 µl | MY09(100ng) | 1.00 µl | GP5+(10pM) | 1.00 µl |
| PC04 (10pM) | 1.00 µl | MY11(100ng) | 1.00 µl | GP6+(10pM) | 1.00 µl |
| 1U/ml Taq | 0.25 µl | 1U/ml Taq | 0.25 µl | 1U/ml Taq | 0.15 µl |
| Mili-Q water | 9.25 µl | Nuclease free water | 4.75 µl | Nuclease free water | 12.35 µl |
| DNA(60ng/µl) | 5.00 µl | DNA(60ng/µl) | 5.00 µl | MY product | 5.00 µl |
| Total reaction volume | 25.00 ml | Total reaction volume | 25.00 ml | Total reaction volume | 25.00 ml |

***Table 4: PCR conditions***

| **Beta-Globin** | | **MY09/MY11** | | **GP5+/GP6+** | |
|---|---|---|---|---|---|
| 1 cycle | 5 min/95°C | 1 cycle | 5 min/95°C | 1 cycle | 5 min/95°C |
| 39 cycles | 30 sec/94°C<br>30 sec/51°C<br>30 sec/72°C | 40 cycles | 1 min/95°C<br>1 min/55°C<br>1 min/72°C | 21 cycles | 1 min/95°C<br>2min/50°C- 40°C (decrement of 0.5°C /cycle)<br>1.5min/72°C |
| 1 cycle | 5 min/72°C | 1 cycle | 10 min/72°C | 10 cycles | 2min/40°C<br>1.5min/72°C |
| - | - | - | - | 1 cycle | 4min/72°C |

***Table 5: List of PCR primer***

| Primers | Sequence (5'-3') | Amplicon (bp) |
|---|---|---|
| HPV Consensus Primers | | |
| MY09 | CGTCCMARRGGAWACTGATC | 450 |
| MY11 | GCMCAGGGWCATAAYAATGG | |
| GP5+ | TTTGTTACTGTGGTAGATACTAC | 150 |
| GP6+ | GAAAAATAAACTGTAAATCATATTC | |
| Beta-Globin primers | | |
| PC03 | ACACAACTGTGTTCACTAGC | 110 |
| PC04 | CAACTTCATCCACGTTCACC | |

### 3.4. RNA *in-situ* hybridization

HPV RNA *in-situ* hybridization **(**RNA-ISH) was performed using the RNAscope ® 2.5 HD assay with Brown HPV HR7 Kit (Advanced Cell Diagnostics Inc. CA, USA) as per manufacturer's protocol. Major steps in brief are outlined.

**3.4.1. Deparaffinization and dehydration:** Freshly cut three five-µm sections (Target, internal positive control, and negative control probes) of tumor tissue were deparaffinized by baking at 60$^{o}$C for 1 hour followed by treatment with xylene and 100% ethanol.

**3.4.2. Pretreatments:** Air-dried sections were subjected to endogenous peroxidase blocking using hydrogen peroxide (Pretreat 1) for 10 min at room temperature followed by washing in distilled water. Sections were then subjected to target retrieval for 15 min in boiling (93$^{o}$C-102$^{o}$C) retrieval solution (Pretreat 2) followed by washing in distilled water and 100% ethanol.

**3.4.3. Protease treatment:** After air drying sections were treated with Protease Plus (Pretreat 3) solution and incubated at 40$^{o}$C for 30 min followed by washing in distilled water.

**3.4.4. Probe hybridization:** Each section was then hybridized with either target probe (HPV HR7 cocktail probe to detect genotype- 16,18, 31, 33, 35, 52, and 58) or an internal positive control probe (Ubiquitin C, a housekeeping gene for confirming adequate RNA quality) or negative control probe (bacterial gene, dapB for confirming the specificity of detection) for at $40^{o}$C in the oven for 2 hr.

**3.4.5. Signal amplification:** A horseradish peroxidase-based signal amplification system was then hybridized to the target probes for signal amplification.

**3.4.6. Signal detection:** Signal detection was done with diaminobenzidine (DAB) for 10 min; the slides were then counterstained with hematoxylin and examined under a microscope (20X - 40X magnifications). For each case, all 3 stained slides (HPV, UBC, and dapB) were examined simultaneously to determine the HPV status.

**3.4.7. Signal evaluation:** Cases with punctate cytoplasmic staining in the positive control section and no staining in the negative control sections were evaluated for HPV target staining. A positive HPV test result was defined as punctate staining that co-localized to the cytoplasm and/or nucleus of the malignant cells.

## 3.5. Immunohistochemistry

### 3.5.1. p16 immunohistochemistry

p16 staining was evaluated by immunohistochemistry (IHC) analysis using CINtec Histology Kit (Roche MTM laboratories AG, Heidelberg, Germany) as per the manufacturer's instructions. Briefly, 5 μm sections of FFPE tumor tissue were deparaffinized by baking at $60^{0}$C for 20 min and treatment with Xylene followed by rehydration through graded alcohol to distilled water. Heat-induced epitope retrieval was performed in preheated retrieval buffer provided in the kit

(pH-9) in a water bath for 18 min, followed by peroxidase blocking with 3% hydrogen peroxide. Sections were then incubated with pre-diluted mouse monoclonal primary antibody (clone E6H4) for 30 min at room temperature (RT), followed by washing step and the treatment with ready to use visualization reagent (polymer reagent conjugated with horseradish peroxidase and goat anti-mouse Fab' antibody fragments) for 30 min at RT. Immunostaining was developed using DAB chromogen solution also provided in the kit. Sections were then counterstained and mounted. Monoclonal mouse anti-Rat oxytocin related neurophysin antibody was used as a negative control reagent provided in the kit. FFPE tissue sections of cervical cancer with strong p16 positive staining were used as a positive control in every run.

### 3.5.2. IHC of other biomarkers

Protein expression levels of total EGFR, pEGFRY1068, pEGFRY1173, HIF1α, CA9, CD44, CD44v6, CD98 (SLC3A2), ALDH1A1, SOX2 and OCT4A were analyzed by IHC using VECTASTATIN® Elite® ABC kit (Vector Laboratories, CA, USA). All the IHC protocols were standardized on positive control tissue showing overexpression of respective proteins to be detected (Table 6). Each IHC batch involved a positive and a negative control.

**3.5.2.1. De-paraffinization and rehydration:** Each lysine coated glass slide consisted of two 5μm thick sections of the same FFPE tumor tissue. One section was the negative control and the other was the test. The sections were baked at 60°C for 20 min and were immediately transferred in Xylene for 15 min at room temperature. Slides were then transferred to Xylene: alcohol (1:1) for 10 min followed by rehydration in 70% ethanol and then 1X phosphate buffer saline (PBS) for 10 min each.

**3.5.2.2. Antigen retrieval:** Heat-induced epitope retrieval was performed in a 700-W microwave oven in the appropriate buffer (Table 6). Slides were allowed to cool to room temperature followed by two washes with fresh 1X PBS for 10 min each.

**3.5.2.3. Blocking of endogenous peroxidase activity:** For blocking the endogenous peroxidase activity, slides were dipped in methanol containing 3% H2O2 for 30 min at room temperature followed by two washes with fresh 1X PBS for 10 min each.

**3.5.2.4. Serum blocking and primary antibody treatment:** Sections were incubated in the normal blocking serum at room temperature for 30 min. After removing excess serum from the section by blotting off on tissue paper, primary antibody (on the test section) and isotype control (on the negative control section at the same dilution as that of primary antibody) or PBS (when polyclonal primary or mouse raised primary was used) were applied and were incubated for 14 hours at $4^{o}$C.

**3.5.2.5. Detection of bound primary antibody:** Next day, sections were washed with PBST (PBS with 0.1% Tween 20) and incubated with biotinylated universal secondary antibody for 40 min at RT followed by washing with PBST and incubation with avidin-biotin-peroxidase for 40 min at RT.

**3.5.2.6. Development and visualization of immunostaining:** After washing, the immunostaining was developed using DAB chromogen. DAB color development was stopped by rinsing the slides with deionized water for 10 min. Sections were counterstained with Mayer's hematoxylin followed by rinsing the slides in deionized water. Tissue sections were dehydrated by passing them through grades of alcohol (70%, 90% and 100%) and xylene. The sections were then mounted with DPX (Merck, Cat#61803502501730). The slides were observed under a light

microscope (AxioImager.Z, Carl Zeiss, GmBH) and images were captured using software Axiovision (Carl Zeiss, GmBH).

***Table 6: List of primary antibodies, retrieval buffer and positive controls used for IHC***

| **Biomarker** | **Primary antibody** | | | **Epitope retrieval buffer** | **Serum blocking** | **Positive control** |
|---|---|---|---|---|---|---|
| | Source | Host species, clone | Dilution | | | |
| **EGFR** | Cell Signaling Tec (#4267) | Rabbit, D38B1 | 1:50 | 10 mM Tris - 1mM EDTA, pH 9 | 1% | Human lung cancer FFPE tissue |
| **pEGFRY1068** | Cell Signaling Tec (#3777) | Rabbit, D7A5 | 1:100 | 1 mM EDTA, pH 8 | 2% | SignalSlide™ Cell Signaling Tec (Cat no- #8102) |
| **pEGFRY1173** | Novus Biologicals (NB110-56948) | Rabbit , E124 | 1:400 | 1 mM EDTA, pH 8 | 2% | SignalSlide™ Cell Signaling Tec (Cat no- #8102) |
| **HIF1α** | Novus Biologicals (NB100-479) | Rabbit polyclonal | 1:300 | 1 mM EDTA, pH 8 | 5% | Human renal cell carcinoma FFPE tissue |
| **CA9** | Cell Signaling Tec (#5649) | Rabbit, D47G3 | 1:100 | 1 mM EDTA, pH 8 | 2% | Human renal cell carcinoma FFPE tissue |
| **CD44** | Novus Biologicals (NBP1-31488) | Rabbit, Polyclonal | 1:500 | 10 mM Tris-1 mM EDTA, pH 9 | 3% | Human normal buccal mucosa |
| **CD44v6** | Novus Biologicals (NBP2-29853) | Mouse, VFF-18 | 1:600 | 1 mM EDTA, pH 8 | 1% | Human normal buccal mucosa |
| **CD98hc** | MBL (BMP090) | Rabbit, Polyclonal | 1:800 | 10mM Sodium citrate, pH-6 | 4% | Human buccal mucosa cancer |
| **ALDH1A1** | abcam (ab52492) | Rabbit, EP1933Y | 1:200 | 1 mM EDTA, pH 8 | 15% | Human stomach cancer |
| **SOX2** | Cell Signaling Tec (#14962) | Rabbit, D1C7J | 1:100 | 10 mM Sodium citrate, pH 6 | 2% | Human normal stomach |
| **OCT4A** | Cell Signaling Tec ( #2890) | Rabbit, C52G3 | 1:100 | 10 mM Sodium citrate, pH 6 | 3% | Human seminoma |

### 3.5.3. Evaluation of IHC staining

IHC staining was evaluated semi-quantitatively by the pathologists who were blinded to HPV-status and patient outcomes. Expression of p16 (nuclear and cytoplasmic) EGFR (membrane or

cytoplasmic), pEGFRY1068 (membrane), pEGFRY1173 (membrane), HIF1α (nuclear), CA9 (membrane), CD44 (complete membrane), CD44v6 (complete membrane), CD98hc (complete membrane), ALDH1A1 (cytoplasmic), SOX2 (nuclear) and Oct4A (nuclear) were assessed by deriving HScore (scale:1–300) using formula, HScore=$\Sigma P_i$ ($i$+1), where $P_i$ is the percentage (0%-100%) of stained tumor cells at each intensity and $i$ is the intensity; $i$=1 (weak), 2 (moderate), 3 (strong). IHC staining of HIF1α and all the CSC markers were independently evaluated by a second pathologist. In the cases with HScore difference of >100 were jointly reevaluated by both the pathologist to obtain the consensus. Biomarkers were analyzed as dichotomized variables. p16 IHC was scored as positive if there was strong as well as diffuse nuclear and cytoplasmic staining present in greater than 70% of the malignant cells, according to the College of American Pathologist (CAP) criteria (102). Due to unavailability of consensus regarding HScore cut-point to be used for dichotomization of all remaining biomarkers, their respective median HScore values were used for categorizing in low/negative and high/positive expression subgroups.

### 3.6. Fluorescence *in situ* hybridization

EGFR gene copy number was assessed by Fluorescence *in situ* hybridization (FISH) using EGFR/CEP7 FISH probe (Abbott Vysis, CA, USA).

**3.6.1. Pretreatment of tissue sections:** Five μm thick FFPE tissue sections were kept at 56°C for 4 h followed by deparaffinization with Xylene at 56°C (twice, for 10 minutes each), followed by two treatments with Xylene for 10 minutes each at room temperature. Slides were dehydrated with 100% ethanol (twice for 5 min each), followed by pretreatment with 2.5% Sodium Thiocyanate for 10 min with slight agitation. Slides were air-dried and treated with 100% ethanol

for 2 min. Retrieval of tissues was done in 10mM Sodium Citrate buffer (pH 5.8) in a microwave at high power (700W) for 4 min.

**3.6.2. Pepsin treatment:** Sections were treated with pepsin working solution (0.2 gm in 50 ml of pre-warmed 10mM hydrochloric acid) and incubated in a humid chamber at 37°C for 40 min. Followed by dehydration of slides in a series of chilled ethanol grades, 70%, 85%, 100% for 2 min each. Slides were dried on the slide warmer for 2-5min.

**3.6.3. Probe hybridization:** For probe hybridization mixture, 1µl of centromere and 1µl of EGFR probe was added to 7µl of FISH hybridization buffer, volume was made up to 10µl using autoclaved MiliQ water (Table 7). Components were thoroughly mixed, and briefly spin in a microcentrifuge. Approximately 5 µl probe (depending on the size of the section) was added to tissues, followed by denaturation of probe DNA and genomic DNA in a humid chamber at 80°C for 5 min and later incubated in a humidified chamber (Neolab, India) at 39°C for overnight hybridization (18-20 h).

***Table 7: Probes used for FISH***

| Probe | Chromosomal region | Probe length |
|---|---|---|
| Vysis LSI EGFR SpectrumOrange | NA | 303 kb |
| CEP 7 SpectrumGreen | 7p11.1-q11.1 | NA |

**3.6.4. Post hybridization washing and counterstaining**

Post-hybridization washes were performed in 2x SSC buffer for 10 min at room temperature and 1.5 M urea/0.1x SSC (pH 7.0–7.5) at 45°C for 15 min. After drying slides were counterstained with Vectashield mounting media containing DAPI (Vector Lab, Cat#H-1200).

### 3.6.5. Visualization and signal enumeration

For visualization of FISH signals, slides were scored using a Zeiss fluorescence microscope (Axioskop II, Germany) equipped with Pinkel filter set 83000 or Metasystems D/G/O/GO/DGO filter set 89084 (cat# H-0650-010-CR) (Chroma technology corporation, VT, USA) along with 63X and 100X objectives. FISH signals were counted in at least 100 nonoverlapping tumor cells under 63X magnification. EGFR gene copy status was classified into five categories depending on the percentage of tumor cells showing different copies of EGFR gene locus and centromere: disomy (≤2 copies in >90% of cells); trisomy (3 copies in ≥10% of cells or ≥4 copies in <10% of cells); low polysomy (≥4 copies in 10%-40% of cells); high polysomy (≥4 copies in ≥40% of cells); and gene amplification (ratio of the EGFR gene to chromosome 7 of ≥2 or ≥15 copies of EGFR per cell in ≥10% of cells). Based on EGFR gene copy status, patients were grouped as FISH negative (disomy, trisomy and low polysomy) or FISH positive (high polysomy and/or EGFR gene amplification) (103).

## 3.7. Statistical analysis

**3.7.1. Descriptive statistics:** Categorical data are presented as frequency and percentage; continuous data are expressed by median and either range or interquartile range (IQR). Bivariate association between categorical variables was analyzed by Pearson's $\chi^2$ test. Differences in continuous variables between the two groups were evaluated by the Mann-Whitney U test.

**3.7.2. Correlation:** Pair wise correlations between continuous variables were assessed using spearman's correlation coefficient.

**3.7.3. Clinical endpoints:** Progression free survival (PFS) was defined as duration from the date of randomization to the date of progression; loco-regional control (LRC) was defined as the

time between the date of randomization and the date of loco-regional failure and OS was calculated as the time from the date of randomization to the date of death (25). PFS, LRC and overall survival (OS) were estimated using Kaplan–Meier method and compared by log-rank tests. Cox proportional hazard models were used to derive hazard ratios (HR) and 95% confidence intervals (CI).

**3.7.4. Analysis of prognostic significance:** The definition used for prognostic and predictive biomarker were as proposed by Clark et al (27). Univariate Cox models were applied to select the most promising biomarkers. A multivariate Cox model using the backward likelihood ratio (LR) method was then applied to adjust for potential confounders (clinical characteristics associated with PFS, LRC, or OS at $P< 0.20$).

**3.7.5. Analysis of predictive significance:** Cox models were constructed for each biomarker expression status (low/negative and high/positive) and treatment effects of NCRT relative to CRT were estimated. Cox models were then fit which included treatments (NCRT versus CRT), biomarker status (low/negative versus high/positive), and the interaction between treatment effect and biomarker status (62, 104).

**3.7.6. Bootstrap resampling method:** Internal validation of prognostic and predictive models was achieved by bootstrap resampling method (1000 samples). Concordance indexes (c-indexes) were also calculated.

**3.7.7. Software used:** Statistical analyses were performed using IBM SPSS software version 21 (SPSS Inc., IL, USA); STATA version 14 (StataCorp, TX, USA) was used for the bootstrap procedure and for generating forest plots.

All reported *P* values are two-sided and *P* value of 0.05 or less was considered statistically significant. The study followed the REMARK guidelines for reporting (105, 106).

# 4.

# Results-I

# Prognostic and predictive significance of HIF1α and EGFR based biomarkers in HPV negative LA-HNSCCs

### 4.1. HPV screening

All the tissue samples with p16 expression (nuclear or cytoplasmic) in >10% tumor cells and/or with DNA-PCR positive result in tissue or saliva were subjected to high risk HPV E6/E7 mRNA in-situ hybridization (RNA-ISH) to confirm the presence of transcriptionally active HPV. A sample was then categorized as HPV positive based on a positive RNA-ISH result. In total, 39/427 (9.1%) cases were p16 positive which includes 22/214 (10.3%) of OPSCC, 9/93 (9.7%) of hypopharyngeal and 8/120 (6.7%) of laryngeal SCC. However, of these only 19 cases tested positive for RNA-ISH; remaining (n=20) p16 positive cases were negative for both RNA-ISH and HPV DNA (Table 8 and Figure 5 A-B).

***Table 8: Results of different HPV screening methods in different major tumor sites***

| **SCC** | **Oropharyngeal** | | **Hypopharyngeal** | | **Laryngeal** | | **Total cases** |
|---|---|---|---|---|---|---|---|
| **RNA-ISH status** | **Negative** | **Positive** | **Negative** | **Positive** | **Negative** | **Positive** | |
| **p16 IHC** | | | | | | | |
| Negative, n (%) | 188 (95.9) | 4 (22.2) | 83 (91.2) | 1 (50) | 111 (96.5) | 1 (20) | 388 (90.9) |
| Positive, n (%) | 8 (4.1) | 14 (77.8) | 8 (8.8) | 1 (50) | 4 (3.5) | 4 (80) | 39 (9.1) |
| Total, n (%) | 196 (100) | 18 (100) | 91 (100) | 2 (100) | 115 (100) | 5 (100) | 427 (100) |
| **DNA-PCR** | | | | | | | |
| Negative, n (%) | 181 (100) | 7 (38.9) | 84 (98.8) | 0 (0) | 109 (99.1) | 1 (25) | 382 (95.5) |
| Positive, n (%) | 0 (0) | 11 (61.1) | 1 (1.2) | 2 (100) | 1 (0.9) | 3 (75) | 18 (4.5) |
| Total, n (%) | 181 (100) | 18 (100) | 85 (100) | 2 (100) | 110 (100) | 4 (100) | 400 (100) |
| **[a]p16 IHC and DNA-PCR** | | | | | | | |
| Negative, n (%) | 181 (100) | 8 (44.4) | 85 (100) | 1 (50) | 110 (100) | 2 (50) | 387 (96.8) |
| Positive, n (%) | 0 (0) | 10 (55.6) | 0 (0) | 1 (50) | 0 (0) | 2 (50) | 13 (3.2) |
| Total, n (%) | 181 (100) | 18 (100) | 85 (100) | 2 (100) | 110 (100) | 4 (100) | 400 (100) |

p16 IHC status is according to CAP (College of American Pathologist) criteria, tumors with ≥70% tumor cells showing moderate to strong diffused nuclear and cytoplasmic staining are called positive; DNA-PCR is nested PCR using primers (MY09/ MY11) and (GP5+/GP6+); [a]Both p16 IHC and DNA-PCR results were not available for all the cases.

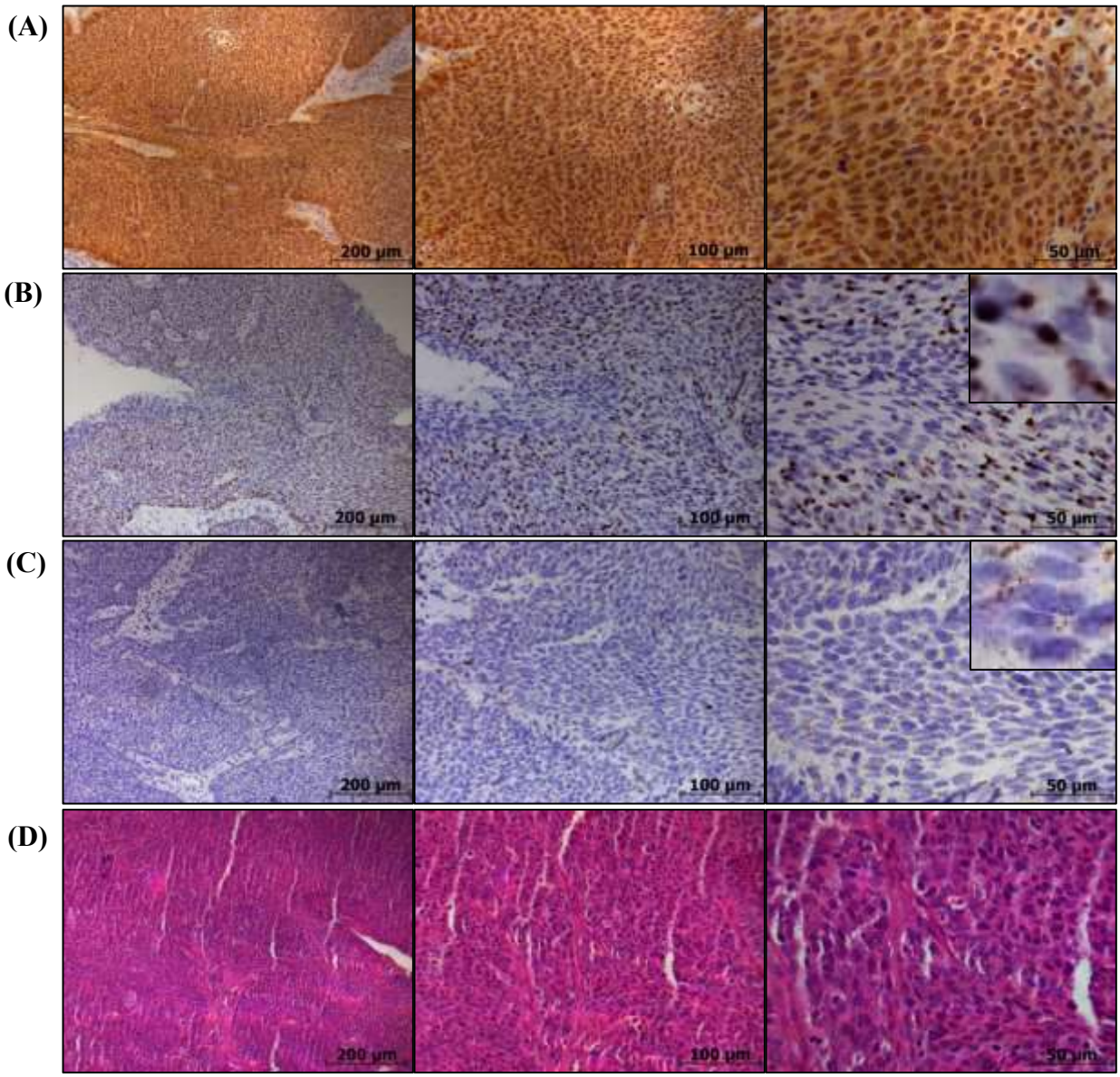



***Figure 5A. HNSCC tumor with p16 positive and HPV positive status.*** *(A) p16 positive IHC (≥70% tumor cells with diffuse moderate nuclear and cytoplasmic staining), (B) Positive high-risk HPV RNA in situ hybridization (RNA-ISH) result (C) Ubiquitin C (RNA-ISH internal positive control) and (D) Hematoxylin and eosin staining.*

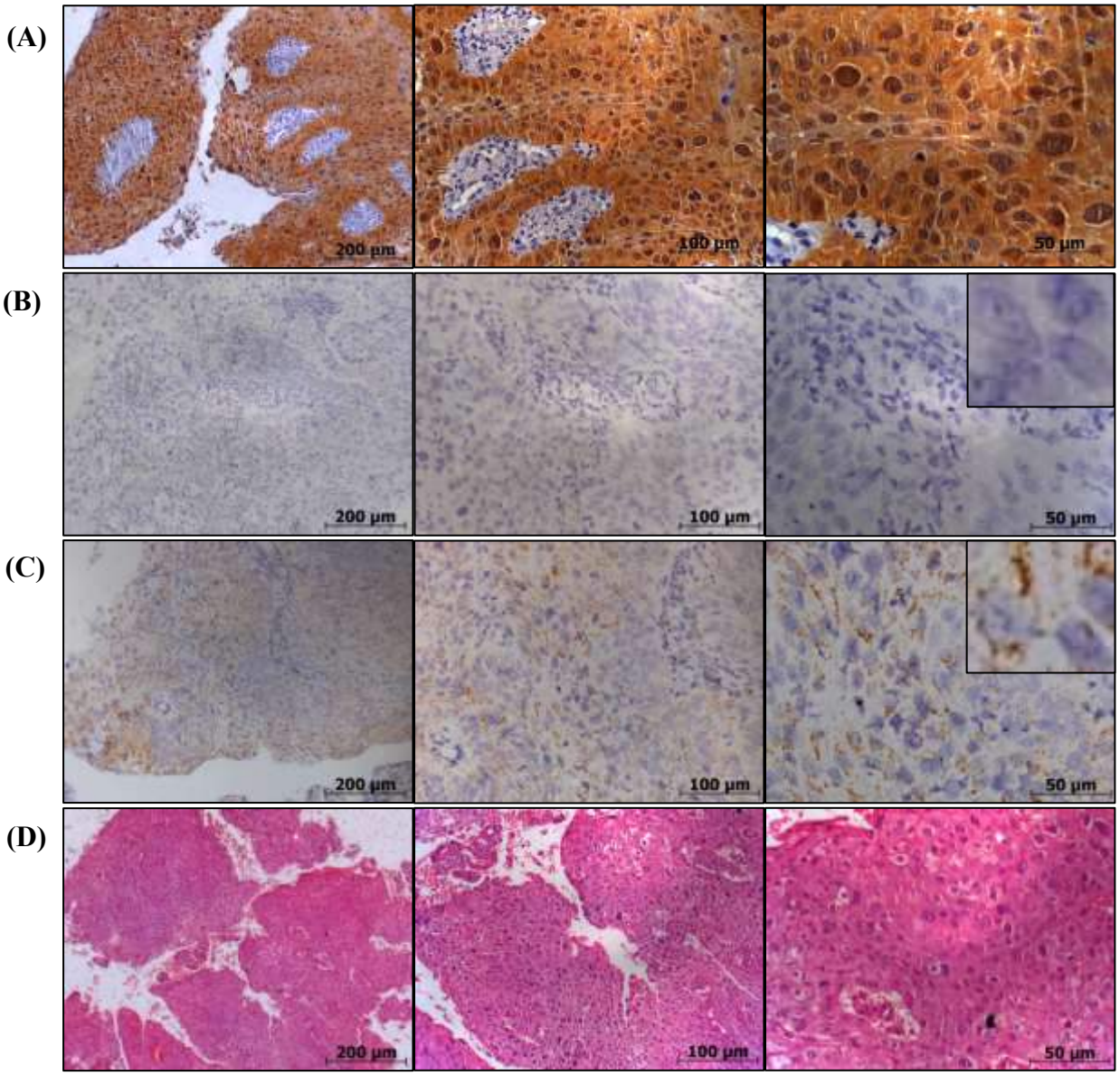
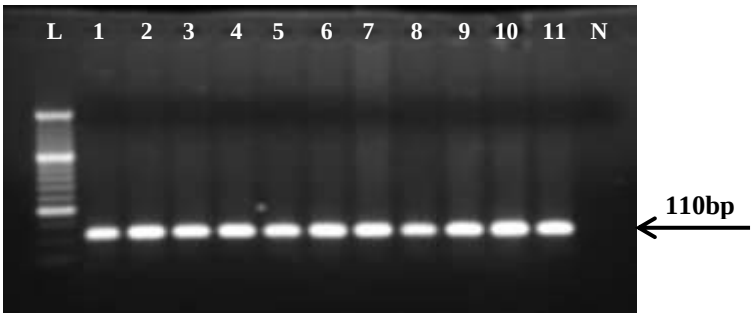



***Figure 5B. HNSCC tumor with p16 positive and HPV negative status****. (A) p16 positive IHC (≥70% tumor cells with diffuse moderate nuclear and cytoplasmic staining), (B) Negative high-risk HPV RNA in situ hybridization (RNA-ISH) result (C) Ubiquitin C (RNA-ISH internal positive control) and (D) Hematoxylin and eosin staining.*

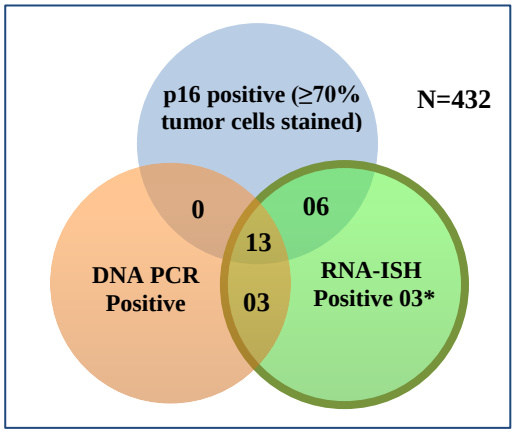
In total 18/400 (4.5%) cases showed the presence of HPV DNA either in saliva and/or FFPE tumor tissue, of which, 11/199 (5.5%) were OPSCC, 3/87 (3.4%) were hypopharyngeal SCC and

4/114 (3.5%) were laryngeal SCC. Out of these 18 HPV-DNA positive cases, 16 were positive for RNA-ISH, the remaining two were negative for p16 staining and RNA-ISH. Representative images of PCR results are given in Figure 6 A-B. In total, 25 (5.8%) cases yielded positive results in RNA-ISH analysis among which 18 (72%) were OPSCC, 2 (8%) were hypopharyngeal SCC and 5 (20%) were laryngeal SCC (Figure 6C). These results indicated that the prevalence of transcriptionally active HPV was low in Indian patients with HNSCC.

**(A) *β-globin PCR results***




**(C)**




**(B) *Nested GP5+/GP6+ PCR results***

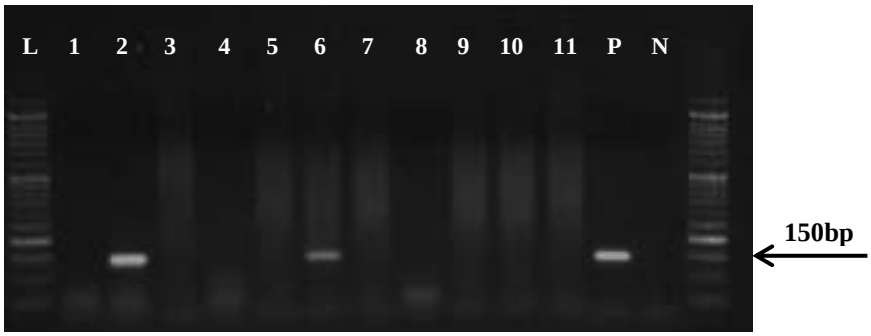



***Figure 6: Representative gel image of β-globin PCR (A), nested GP5+/GP6+ PCR (B) result. Venn diagram showing how 25 HPV positive cases were derived (C).***

*Lanes,1-11: HNSCC samples; L: GeneRuler 50 bp DNA Ladder (Thermo Scientific). N: No template control; P: DNA of HeLa (HPV-18) cervical cell line, used as a positive control; Specific band indicated (arrow) at 110bp and 150bp for β-globin and HPV positivity respectively. Sample 2 and 6 shows the presence of HPV-DNA. (*) RNA-ISH positive cases showing p16 staining in <70% tumor cells (p16 negative cases)*

## 4.2. Study groups and patients' baseline clinical and demographic parameters

We excluded 25 HPV (RNA-ISH) positive cases and additional 3 cases in which HPV RNA-ISH results were inconclusive. We then carried out prognostic and predictive biomarker analysis in the remaining four hundred and four HPV negative cases, out of which 206 received CRT and 198 received NCRT treatment. The median follow-up of the patients was 39.13 months; four-year survival rates are reported. Out of 404 patients included in the study, a total of 195 patients (48.3%) had died, 188 (46.5%) progressed and 155 (38.4%) experienced loco-regional failure at the time of analysis. The treatment outcomes in the biomarker subgroup (n=404) are provided in Figure 7. Patients in NCRT group (n=198) had significantly improved PFS [HR (95% CI)= 0.67 (0.51-0.90), 52.6 vs 41.9 months, *P* (log-rank)= 0.007] and LRC [HR (95%CI)= 0.66 (0.48-0.90), 59.4 vs 49.4 months, *P* (log-rank)= 0.009] and also showed a trend towards longer OS [HR (95%CI)= 0.78 (0.59-1.03), 47.7 vs 42.3 months, *P* (log-rank)= 0.081] compared to patients in CRT treatment group (n=206). Baseline characteristics of the patients were balanced between the two treatment groups (Table 9).

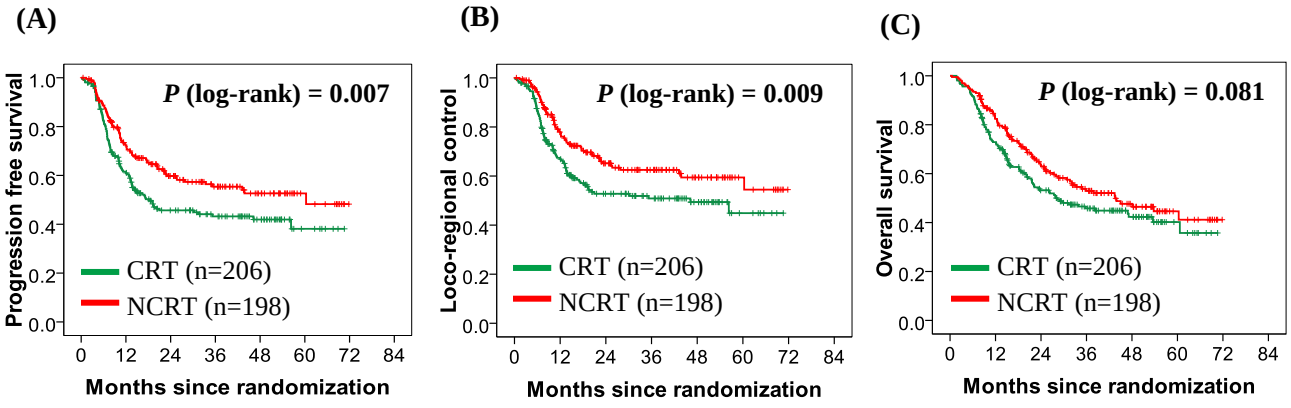



***Figure 7: Kaplan-Meier plots showing clinical outcomes in both the treatment groups.***

*Progression free survival (A), Loco-regional control (B) and Overall survival (C)*

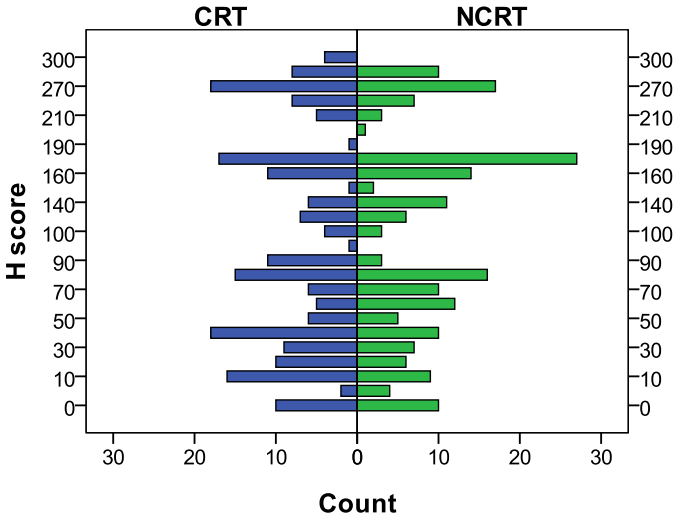
***Table 9: Demographics and baseline characteristics of HNSCC patients enrolled in a randomized clinical trial, CTRI/2014/09/004980, Tata Memorial Hospital, India***

| Characteristics | Trial population (N=536) | | Biomarker subgroup (n=404) | | |
|---|---|---|---|---|---|
| | CRT (n=268) | NCRT (n=268) | CRT (n=206) | NCRT (n=198) | *P* value |
| **Age (Years)** | | | | | |
| Median & range | 54(26-77) | 55(20-73) | 54(28-77) | 56(23-73) | |
| 40 or below | 26(9.7) | 30(11.2) | 16(7.8) | 19(9.6) | |
| >40 and <60 | 165(61.6) | 156(58.2) | 132(64.1) | 110(55.6) | 0.217 |
| 60 and above | 77(28.7) | 82(30.6) | 58(28.1) | 69(34.8) | |
| **Gender** | | | | | |
| Male | 231(86.2) | 226(84.3) | 181(88.3) | 171(86.4) | 0.653 |
| Female | 37(13.8) | 42(15.7) | 25(11.7) | 27(13.6) | |
| **ECOG PS** | | | | | |
| 0 | 58(21.6) | 60(22.4) | 47(22.8) | 44(22.2) | 0.887 |
| 1-2 | 210(78.4) | 208(77.6) | 159(77.2) | 154(77.8) | |
| **Site of tumor** | | | | | |
| Hypopharynx | 47(17.5) | 62(23.1) | 42(20.4) | 49(24.7) | |
| Larynx | 83(31) | 72(26.9) | 66(32) | 49(24.7) | 0.174 |
| Oral Cavity | 3(1.1) | 0(0) | 2(1) | 0(0) | |
| Oropharynx | 135(50.4) | 134(50) | 96(46.6) | 100(50.5) | |
| **Clinical stage[a]** | | | | | |
| II | 5 (1.9) | 4 (1.5) | 0 (0) | 0 (0) | |
| III | 77 (28.7) | 65 (24.3) | 58 (28.2) | 40 (20.2) | 0.158 |
| IVA | 80 (29.9) | 81 (30.2) | 57 (27.7) | 65 (32.8) | |
| IVB | 106 (39.6) | 118 (44.0) | 91 (44.2) | 93 (47.0) | |
| **T stage[a]** | | | | | |
| T1-T2 | 56 (20.9) | 41 (15.3) | 41 (19.9) | 34 (17.2) | 0.48 |
| T3-T4 | 212 (79.1) | 227 (84.7) | 165 (80.1) | 164 (82.8) | |
| **N stage[a]** | | | | | |
| N0-N1 | 107 (39.9) | 92 (34.3) | 80 (38.8) | 64 (32.3) | 0.172 |
| N2-N3 | 161 (60.1) | 176 (65.7) | 126 (61.2) | 134 (67.7) | |
| **Tobacco and alcohol habits** | | | | | |
| No habits | 27(10.1) | 30(11.2) | 14(6.8) | 16(8.1) | |
| Exclusive chewer | 44(16.4) | 48(17.9) | 36(17.5) | 40(20.2) | |
| Exclusive smoker[b] | 50(18.6) | 49(18.3) | 37(18) | 33(16.7) | 0.513 |
| Exclusive drinker | 3(1.1) | 8(3) | 1(0.5) | 4(2) | |
| Mixed habits [c] | 139(51.9) | 121(45.1) | 114(55.3) | 98(49.5) | |
| No information | 5(1.9) | 12(4.5) | 4(1.9) | 7(3.5) | |

Data are number (%) unless otherwise indicated. CRT=cisplatin-radiation; NCRT=nimotuzumab plus cisplatin-radiation; ECOG=Eastern Cooperative Oncology Group ([a]) According to AJCC-UICC system (8$^{th}$ edition); ([b]) Bidi or cigarette smoking; ([c]) Tobacco chewing along with bidi/cigarette smoking and/or alcohol drinking; p value, Pearson Chi square test is comparing between CRT and NCRT patients in the biomarker subgroup.

### 4.3. Expression of hypoxia inducible factor 1α

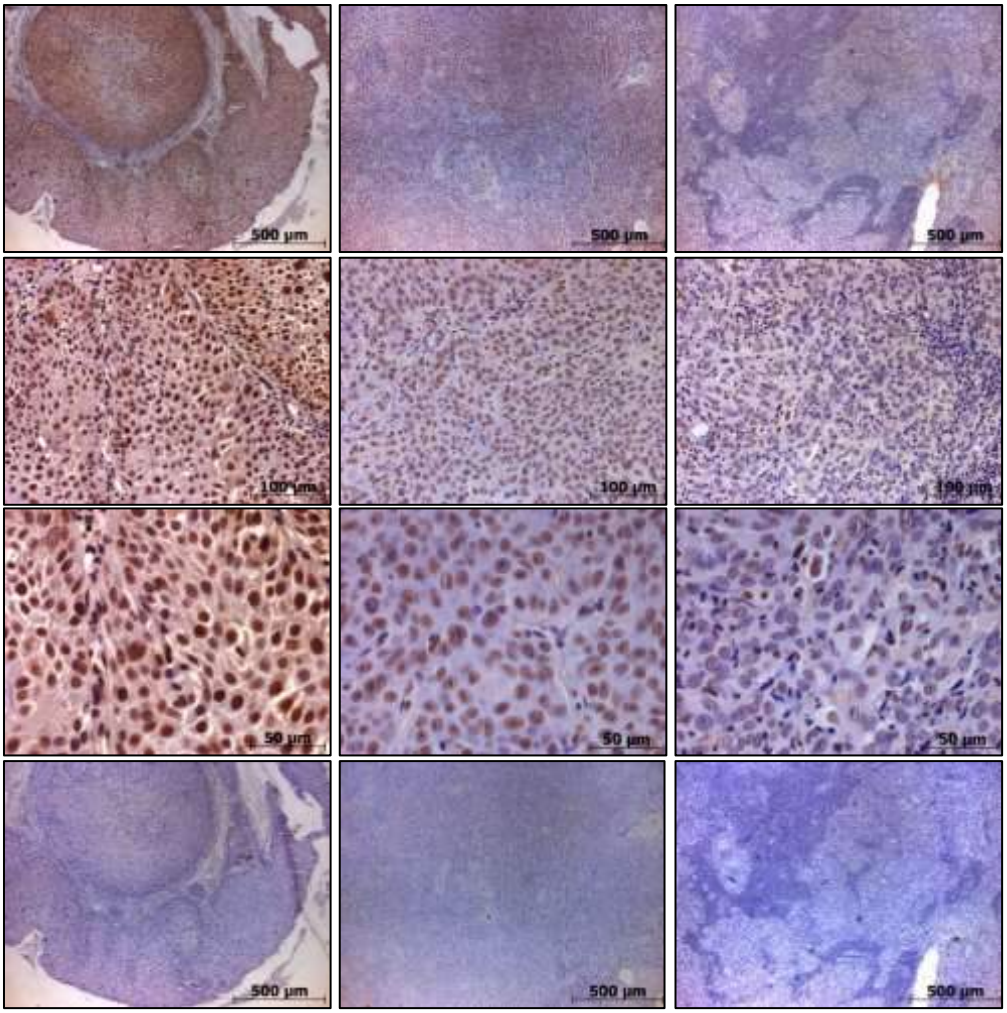
We have analyzed the nuclear expression of HIF1α in total of 392 samples. Out of which, 20 cases (5.1 %) were completely negative (HScore=0) for HIF1α expression. Weak, moderate, and strong nuclear staining was observed in a total of 136 (34.7%), 131 (33.4%), and 105 (26.8%) cases respectively. Out of 392 cases analyzed for expression, 228 (58.2%) cases showed HIF1α staining >50% of the tumor cells. Based on median HScore value, patients were categorized in low expressing (HScore ≤90) or high expressing groups (HScore >90). Frequency distributions of HScore in both the treatment groups were comparable and are provided in Figure 8A. Representative IHC staining images of low and high expression are given in Figure 8B.




| CRT | NCRT |
|---|---|
| Median=90<br>IQR=40-180<br>n=199 | Median=120<br>IQR=50-180<br>n=193 |

***Figure 8A: Histograms showing frequency distribution of nuclear HIF1α expression.***

*IQR= inter quartile range.*




**HScore=270** **HScore=180** **HScore=70**

***Figure 8B: Representative IHC staining results showing nuclear expression of HIF1α.*** *Bottom most panel shows the respective negative control for each case.*

Immunostaining of HIF1α was independently evaluated by a second pathologist (NM), the agreement between the scoring of two pathologists (SR and NM) is shown using the Bland-Altman plot (Figure 9). Concordance correlation coefficient for the same was 0.89 (0.87-0.91) (107, 108).

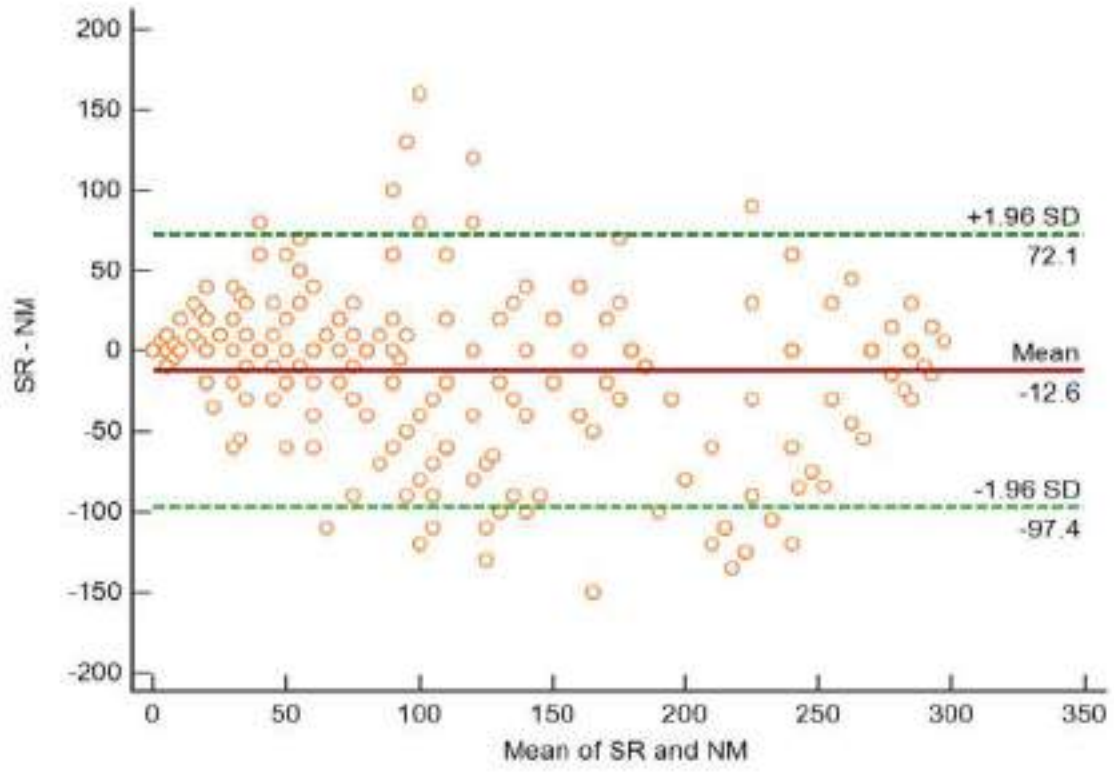


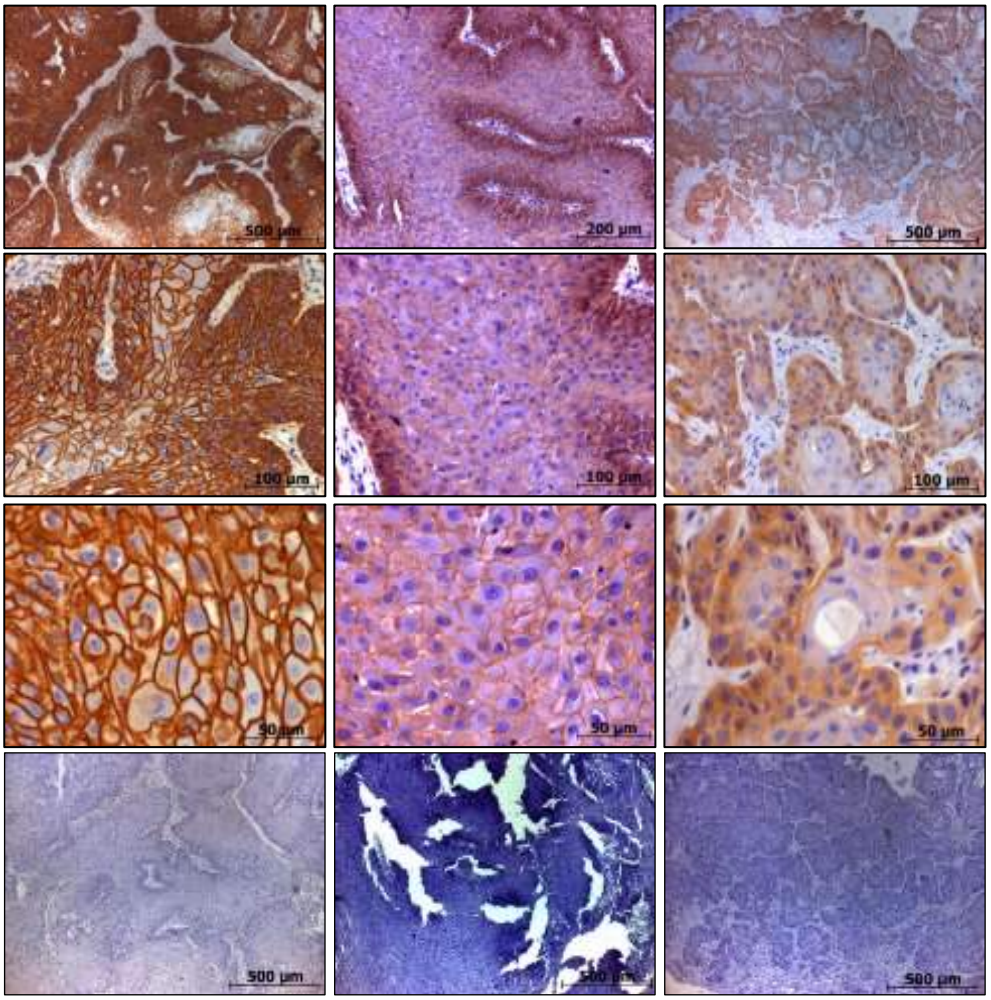

***Figure 9: Bland-Altman plot showing agreement between HIF1α scoring of two pathologists****. The vertical axis indicates the difference between the two pathologist's (SR and NM) HScore and the horizontal axis is the average of the two. The solid red line represents the mean value and the dashed green show ±1.96 standard deviation (SD).*

## 4.4. Expression of epidermal growth factor receptor based biomarkers

### 4.4.1. Expression of total EGFR

We have analyzed both membrane and cytoplasmic expression of EGFR in 404 patients. The respective representative images of IHC staining are shown in Figure 10A-B**.** The EGFR membrane expression was not detected in 43 (10.6%) cases; however, only 2 (0.5%) cases

showed the absence of cytoplasmic EGFR staining. Weak, moderate, and strong membrane EGFR staining was observed in 23 (5.7%), 277 (68.6%), and 61 (15.1%) cases respectively.

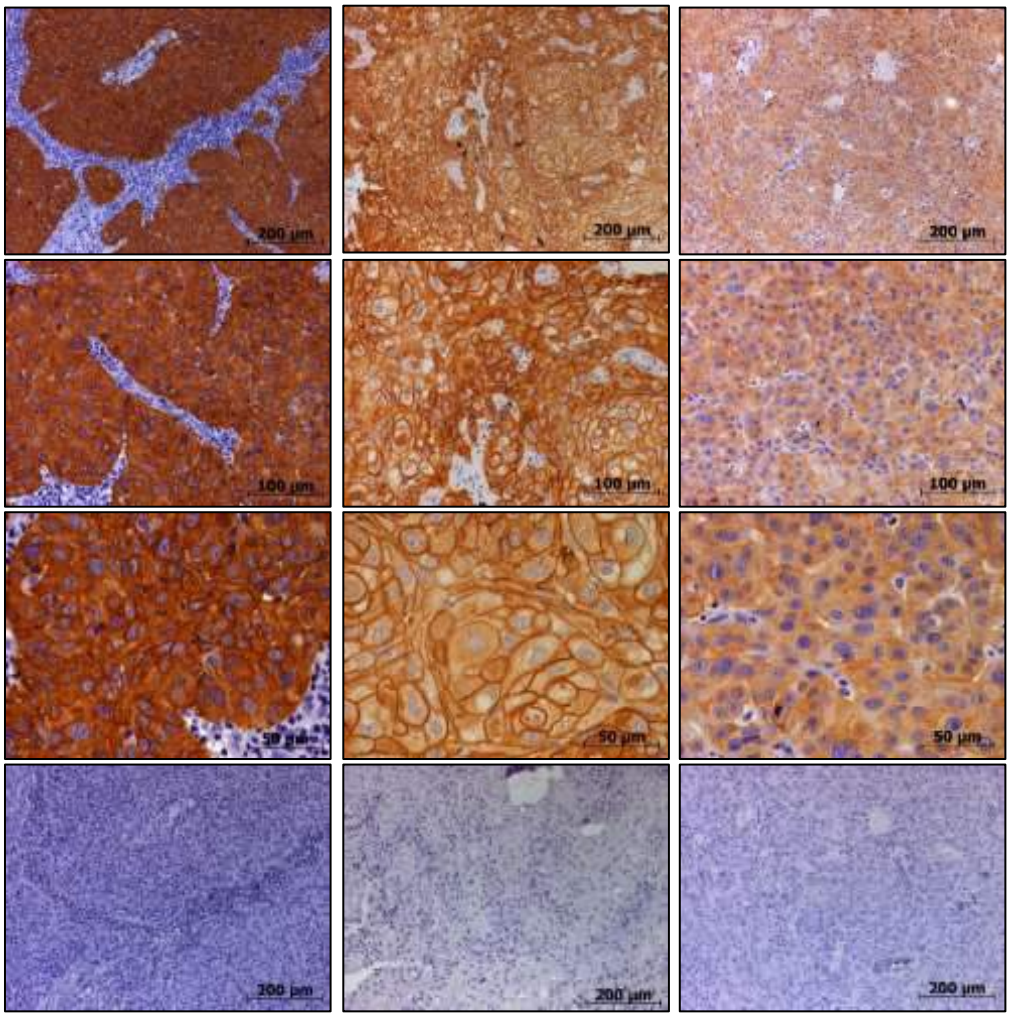



**HScore=285** **HScore=120** **HScore=95**

***Figure 10A: Representative IHC staining results showing membrane expression of EGFR****. Bottom panel shows the respective isotype control for each case.*

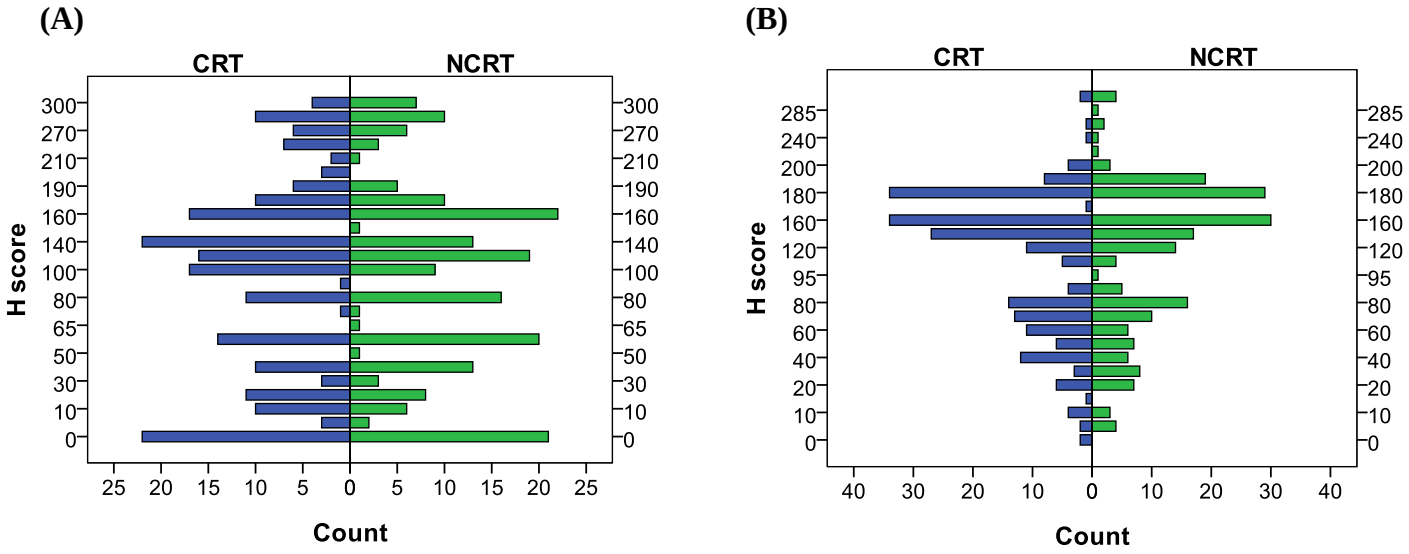
Similarly, cytoplasmic expression was observed in 127 (31.4%), 260 (64.4%), and 15 (3.7%) cases respectively. In total 204 (50.5%) and 311 (77%), cases showed membrane and cytoplasmic expression in >50% tumor cells respectively. Based on median HScore value

(membrane, median HScore=100; cytoplasmic, median HScore=140) patients were categorized in low or high expression groups (Figure 10 A-B). The frequency distribution of membrane and cytoplasmic EGFR HScore in both treatment groups were comparable and are provided in Figure 11 A-B.




**(HScore =300)** **(HScore=180)** **(HScore=95)**

***Figure 10B: Representative IHC staining results showing cytoplasmic expression of EGFR****. Bottom panel shows the respective isotype control for each case.*



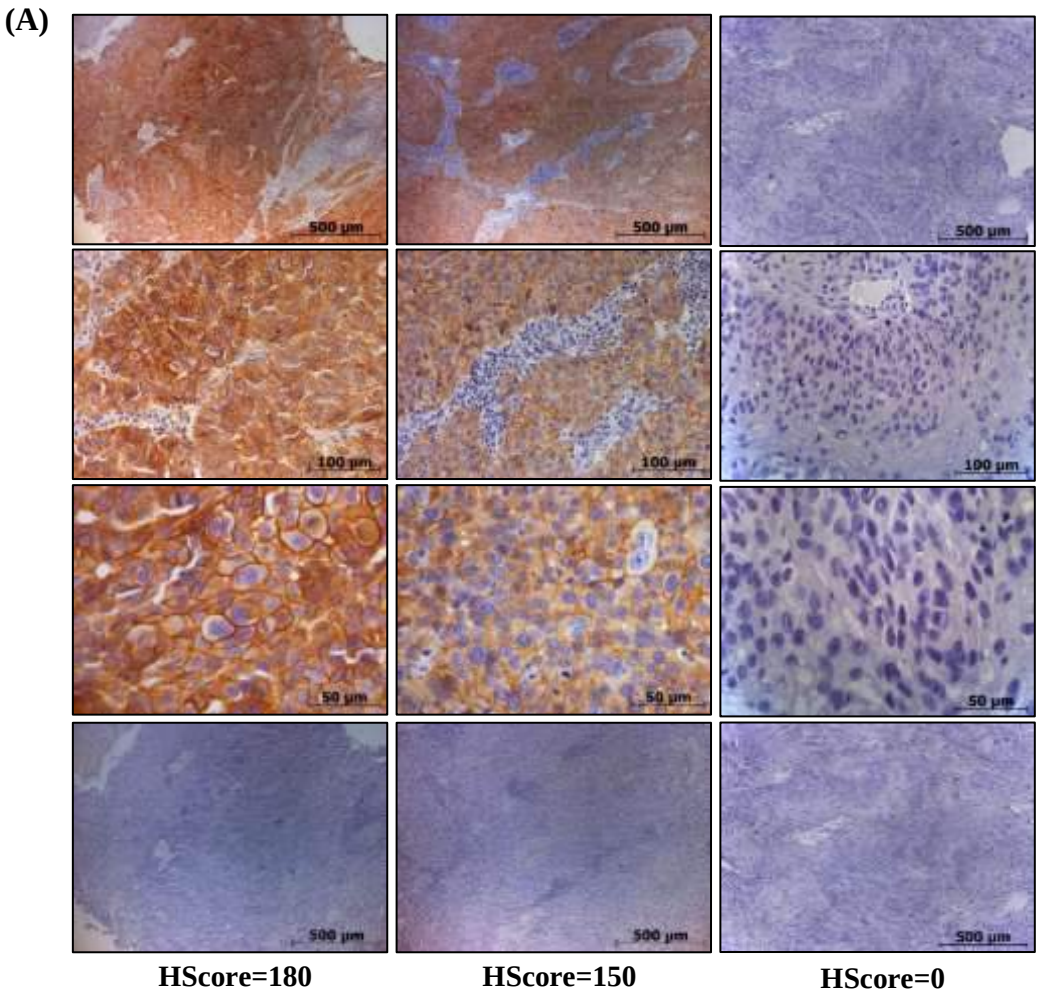

| EGFR (membrane) | |
|---|---|
| Median=110<br>IQR=40-160<br>n=206 | Median=100<br>IQR=40-160<br>n=198 |

| EGFR (cytoplasm) | |
|---|---|
| Median=140<br>IQR=70-163<br>n=206 | Median=140<br>IQR=70-180<br>n=198 |

***Figure 11: Histograms showing frequency distribution of EGFR expression****. Membrane (A) and cytoplasmic (B) expression across both the treatment groups. IQR= inter quartile range.*

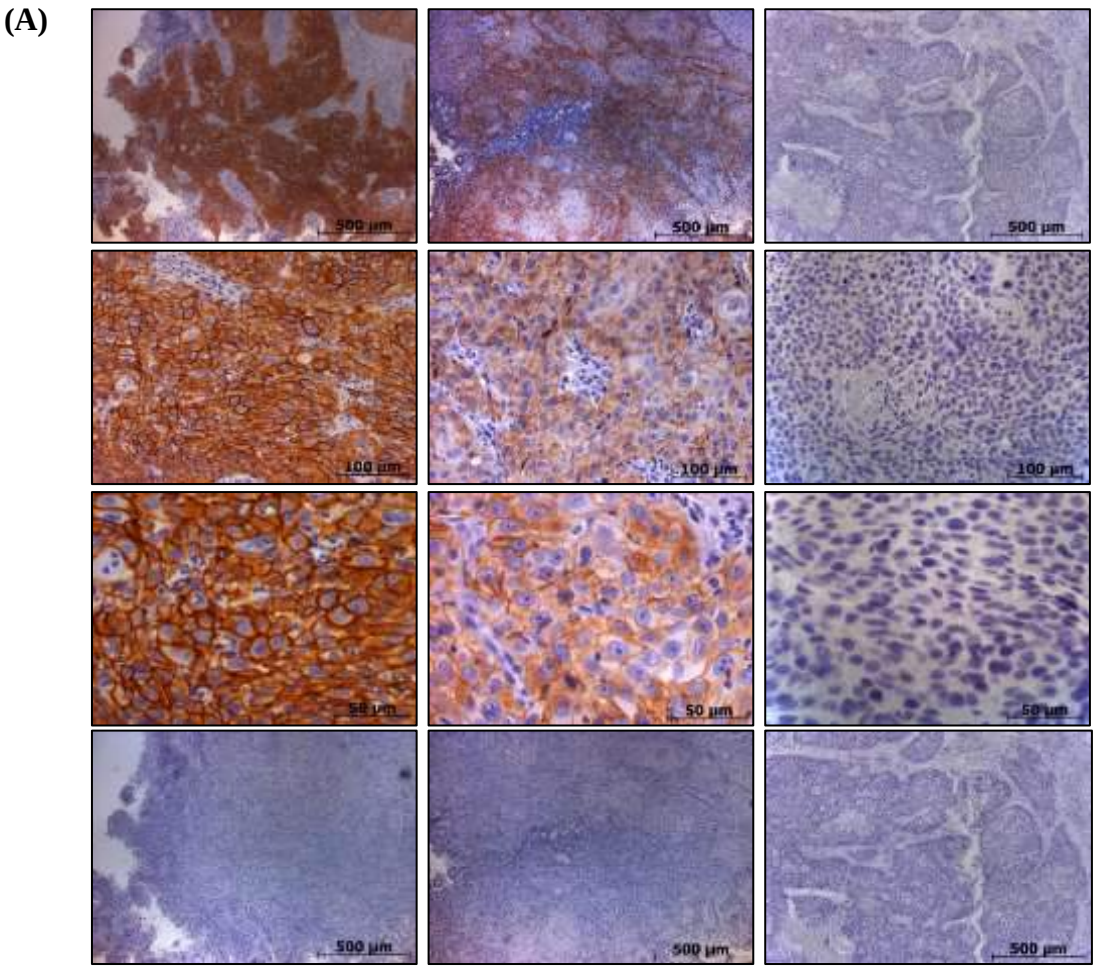
### 4.4.2. Expression of pEGFR dimers

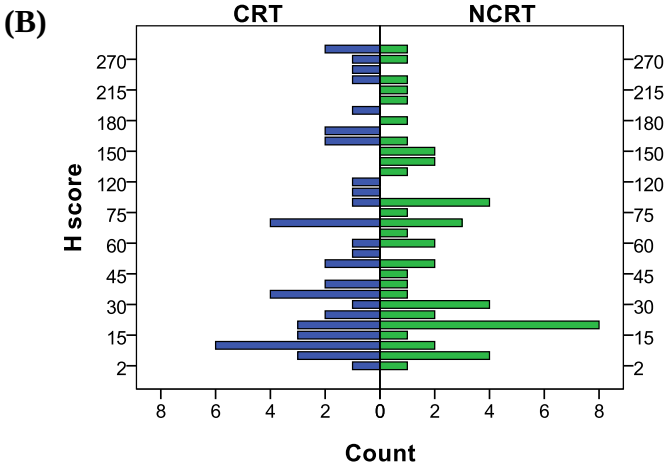
We have analyzed the membrane expression of EGFR phospho dimers- pEGFRY1068 and pEGFRY1173. Representative images of IHC staining and frequency distribution of HScore (>0) in both the treatment groups of pEGFRY1068 and pEGFRY1173 dimes are given in Figures 12 and 13 respectively. Overall, the expression of pEGFRY1068 and pEGFRY1173 showed a skewed distribution as >80% and >70% of the cases respectively were negative (HScore=0) in both treatment groups.




| pEGFRY1068 (membrane) | |
|---|---|
| Median=50<br>IQR=15-87<br>n=32 | Median=40<br>IQR=10-85<br>n=33 |

***Figure 12: Representative IHC staining (A) and frequency distribution (B) of membrane pEGFRY1068 expression.*** *Bottom IHC image panel shows respective isotype control. IQR= inter quartile range.*




| pEGFRY1173 (membrane) | |
|---|---|
| Median=37.5<br>IQR=15-116<br>n=46 | Median=47.5<br>IQR=20-92.5<br>n=50 |

***Figure 13: Representative IHC staining (A) and frequency distribution (B) of membrane pEGFRY1173 expression.*** *Bottom IHC image panel shows respective isotype control. IQR= inter quartile range.*

### 4.4.3. EGFR gene copy number change

EGFR gene copy number change was determined by FISH. EGFR gene copy status was classified into five categories depending on the percentage of tumor cells showing different copies of EGFR gene locus and centromere: disomy, trisomy, low polysomy, high polysomy, and gene amplification. Representative images of FISH results are provided in Figure 14. The distribution of the patients in these categories was comparable between the two treatment groups (Table 10).

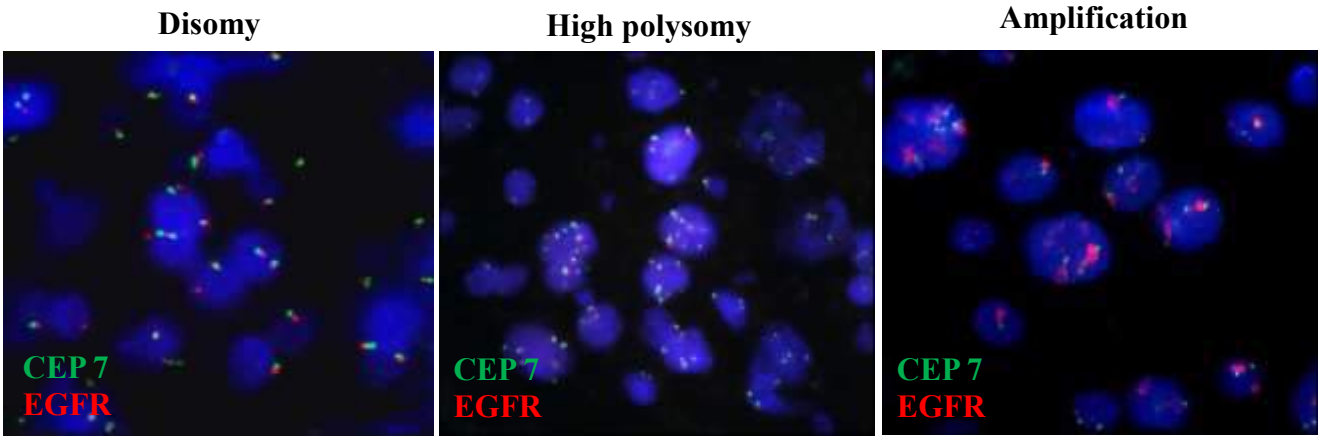



***Figure 14: Representative EGFR FISH images showing EGFR gene copy number change.*** *Disomy [≤2 copies in >90% of cells], high polysomy [≥4 copies in ≥40% of cells] and gene amplification which can be seen as large clusters of EGFR signals*

***Table 10: EGFR-FISH signal categorization and distribution of the patients***

| Categories | Definition | CRT (n=143) | NCRT (n=148) |
|---|---|---|---|
| Disomy | ≤2 copies in >90% cells | 6 (4.2) | 6 (4.0) |
| Trisomy | 3 copies in ≥10% cells or ≥4 copies in <10% cells | 5 (3.5) | 5 (3.4) |
| Low polysomy | ≥4 copies in 10% -40% cells | 84 (58.7) | 90 (60.8) |
| High polysomy | ≥4 copies in ≥40% cells | 14 (9.8) | 17 (11.5) |
| Amplification | ratio of the EGFR gene to chromosome 7 of ≥2, or ≥15 copies of EGFR per cell in ≥10% of cells | 34 (23.8) | 30 (20.3) |

*Pearson's $\chi 2$ Square test; Data are number (%); Disomy, trisomy and low polysomy are categorized as EGFR FISH negative; high polysomy and amplification are categorized as EGFR FISH positive.

## 4.5. Correlation among different biomarkers

The correlation among biomarker expression was analyzed using HScore values (Table 11A) and after dichotomization of the biomarker expression (Table 11B).

***Table 11A: Correlation among different biomarkers (continuous HScore)***

| | | EGFR (Cytoplasm) | pEGFRY1068 | pEGFRY1173 | HIF1α |
|---|---|---|---|---|---|
| EGFR (Membrane) | R | .61** | .29** | .33** | .17* |
| | *P* | 0 | 0 | 0 | 0.016 |
| | n | 206 | 200 | 188 | 199 |
| EGFR (Cytoplasm) | R | | .24** | .27** | -0.02 |
| | *P* | | 0.001 | 0 | 0.776 |
| | n | | 200 | 188 | 199 |
| pEGFRY1068 | R | | | .64** | -0.02 |
| | *P* | | | 0 | 0.814 |
| | n | | | 188 | 195 |
| pEGFRY1173 | R | | | | 0.10 |
| | *P* | | | | 0.17 |
| | n | | | | 185 |

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed). Rho= Spearman's correlation coefficient; n= no of samples

***Table 11B: Correlation among different biomarkers (categorical)***

| | | EGFR (Membrane) | | EGFR (Cytoplasm) | | pEGFRY1068 | | pEGFRY1173 | | EGFR-FISH | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Low** | **High** | **Low** | **High** | **Negative** | **Positive** | **Negative** | **Positive** | **Negative** | **Positive** |
| **HIF1α** | Low, n (%) | 115 (57.5) | 85 (42.5) | 108 (54) | 92 (46) | 167 (85.2) | 29 (14.8) | 147 (77.8) | 42 (22.2) | 87 (62.1) | 53 (37.9) |
| | High, n (%) | 82 (42.7) | 110 (57.3) | 113 (58.9) | 79 (41.1) | 152 (81.3) | 35 (18.7) | 127 (70.2) | 54 (29.8) | 107 (71.8) | 42 (28.2) |
| | *P** | 0.003 | | 0.333 | | 0.304 | | 0.095 | | 0.080 | |
| | R | 0.15 | | -0.049 | | 0.053 | | 0.087 | | -0.10 | |
| **EGFR (Membrane)** | Low, n (%) | | | 156 (76.5) | 48 (23.5) | 182 (92.9) | 14 (7.1) | 164 (86.3) | 26 (13.7) | 110 (73.8) | 39 (26.2) |
| | High, n (%) | | | 73 (36.5) | 127 (63.5) | 146 (74.1) | 51 (25.9) | 116 (62.4) | 70 (37.6) | 86 (60.6) | 56 (39.4) |
| | *P** | | | 0.000 | | 0.000 | | 0.000 | | 0.016 | |
| | R | | | 0.40 | | 0.25 | | 0.28 | | 0.14 | |
| **EGFR (Cytoplasm)** | Low, n (%) | | | | | 198 (89.6) | 23 (10.4) | 167 (79.9) | 42 (20.1) | 119 (72.6) | 45 (27.4) |
| | High, n (%) | | | | | 130 (75.6) | 42 (24.4) | 113 (67.7) | 54 (32.3) | 77 (60.6) | 50 (39.4) |
| | *P** | | | | | 0.000 | | 0.007 | | 0.031 | |
| | R | | | | | 0.19 | | 0.14 | | 0.13 | |
| **pEGFRY1068** | Negative, n(%) | | | | | | | 269 (85.4) | 46 (14.6) | 178 (73.6) | 64 (26.4) |
| | Positive, n(%) | | | | | | | 11 (18) | 50 (82) | 16 (35.6) | 29 (64.4) |
| | *P** | | | | | | | 0.000 | | 0.000 | |
| | R | | | | | | | 0.57 | | 0.30 | |
| **pEGFRY1173** | Negative, n(%) | | | | | | | | | 158 (74.5) | 54 (25.5) |
| | Positive, n (%) | | | | | | | | | 29 (44.6) | 36 (55.4) |
| | *P** | | | | | | | | | 0.000 | |
| | R | | | | | | | | | 0.27 | |

(*) Pearson's $\chi 2$ tests; R=Pearson correlation coefficient

A moderate positive correlation was observed between the membrane and cytoplasmic expression of EGFR (Rho=0.61) as well as between pEGFRY1068 and pEGFRY1173 (Rho=0.64). Both membrane and cytoplasmic EGFR expression showed a weak positive correlation with pEGFR dimers (Figure 15). A very weak positive correlation was also observed between HIF1α and membrane EGFR expression (Rho=0.17).

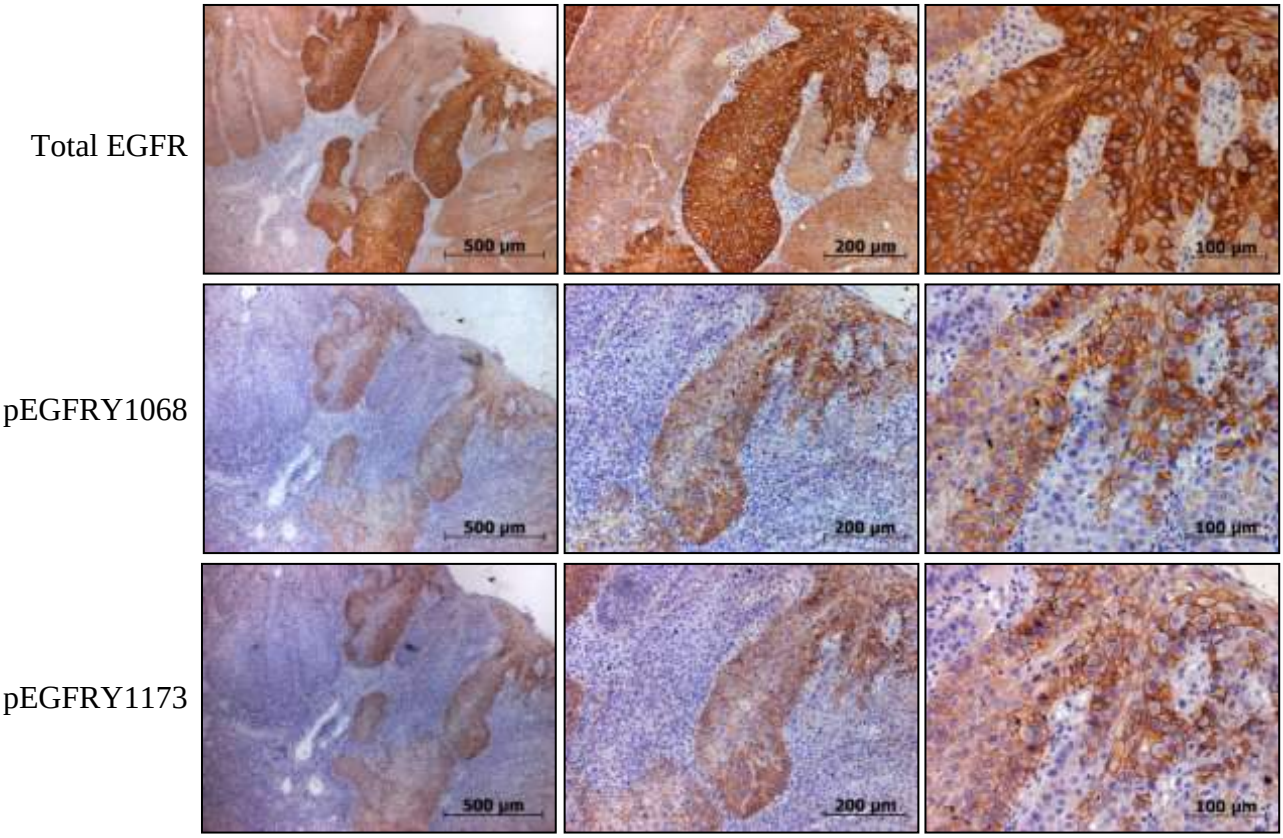



***Figure 15: Tumor region with high total EGFR expression showed positive expression of pEGFR dimers- pEGFRY1068 and pEGFRY1173.***

### 4.6. Association between biomarker status and patients' baseline parameters

Cytoplasmic EGFR status was associated with a higher disease stage (*P*=0.027, Table 12). We did not observe any other significant association between biomarker and baseline characteristics.

***Table 12: Association between biomarkers and patient's baseline clinical-demographic parameters***

| | | Age (Years) | | Gender | | Tobacco-alcohol habit | | Site of tumor | | Clinical stage | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Below 60 | Above 60 | Male | Female | No habit | With habit | Oropharynx | Others | III | IV |
| HIF1α | Low, n (%) | 136 (68.0) | 64 (32.0) | 171 (85.5) | 29 (14.5) | 10 (5.1) | 185 (94.9) | 94 (47.0) | 106 (53.0) | 43 (21.5) | 157 (78.5) |
| | High, n (%) | 130 (67.7) | 62 (32.3) | 172 (89.6) | 20 (10.4) | 17 (9.0) | 171 (91.0) | 93 (48.4) | 99 (51.6) | 52 (27.1) | 140 (72.9) |
| | *P** | 0.951 | | 0.222 | | 0.135 | | 0.776 | | 0.197 | |
| EGFR (Membrane) | Low, n (%) | 139 (68.1) | 65 (31.9) | 184 (90.2) | 20 (9.8) | 14 (7.1) | 184 (92.9) | 99 (48.5) | 105 (51.5) | 47 (23.0) | 157 (77.0) |
| | High, n (%) | 138 (69.0) | 62 (31.0) | 168 (84.0) | 32 (16.0) | 16 (8.20) | 179 (91.8) | 97 (48.5) | 103 (51.5) | 51 (25.5) | 149 (74.5) |
| | *P** | 0.852 | | 0.063 | | 0.672 | | 0.995 | | 0.564 | |
| EGFR (Cytoplasm) | Low, n (%) | 157 (68.6) | 72 (31.4) | 204 (89.1) | 25 (10.9) | 16 (7.2) | 205 (92.8) | 111 (48.5) | 118 (51.5) | 65 (28.4) | 164 (71.6) |
| | High, n (%) | 120 (68.6) | 55 (31.4) | 148 (84.6) | 27 (15.4) | 14 (8.1) | 158 (91.9) | 85 (48.6) | 90 (51.4) | 33 (18.9) | 142 (81.1) |
| | *P** | 0.998 | | 0.18 | | 0.739 | | 0.984 | | 0.027 | |
| pEGFR Y1068 | Negative, n (%) | 228 (69.5) | 100 (30.5) | 285 (86.9) | 43 (13.1) | 27 (8.5) | 291 (91.5) | 162 (49.4) | 166 (50.6) | 78 (23.8) | 250 (76.2) |
| | Positive, n (%) | 42 (64.6) | 23 (35.4) | 56 (86.2) | 9 (13.8) | 3 (4.7) | 61 (95.3) | 30 (46.2) | 35 (53.8) | 17 (26.2) | 48 (73.8) |
| | *P** | 0.437 | | 0.873 | | 0.302 | | 0.633 | | 0.683 | |
| pEGFR Y1173 | Negative, n (%) | 193 (68.9) | 87 (31.1) | 240 (85.7) | 40 (14.3) | 23 (8.5) | 249 (91.5) | 133 (47.5) | 147 (52.5) | 71 (25.4) | 209 (74.6) |
| | Positive, n (%) | 65 (67.7) | 31 (32.3) | 86 (89.6) | 10 (10.4) | 6 (6.3) | 89 (93.7) | 52 (54.2) | 44 (45.8) | 19 (19.8) | 77 (80.2) |
| | *P** | 0.824 | | 0.335 | | 0.506 | | 0.26 | | 0.27 | |
| EGFR-FISH | Negative, n (%) | 137 (69.9) | 59 (30.1) | 167 (85.2) | 29 (14.8) | 19 (9.7) | 176 (90.3) | 99 (50.5) | 97 (49.5) | 47 (24.0) | 149 (76.0) |
| | Positive, n (%) | 63 (66.3) | 32 (33.7) | 83 (87.4) | 12 (12.6) | 4 (4.3) | 90 (95.7) | 41 (43.2) | 54 (56.8) | 22 (23.2) | 73 (76.8) |
| | *P** | 0.537 | | 0.619 | | 0.43 | | 0.239 | | 0.877 | |

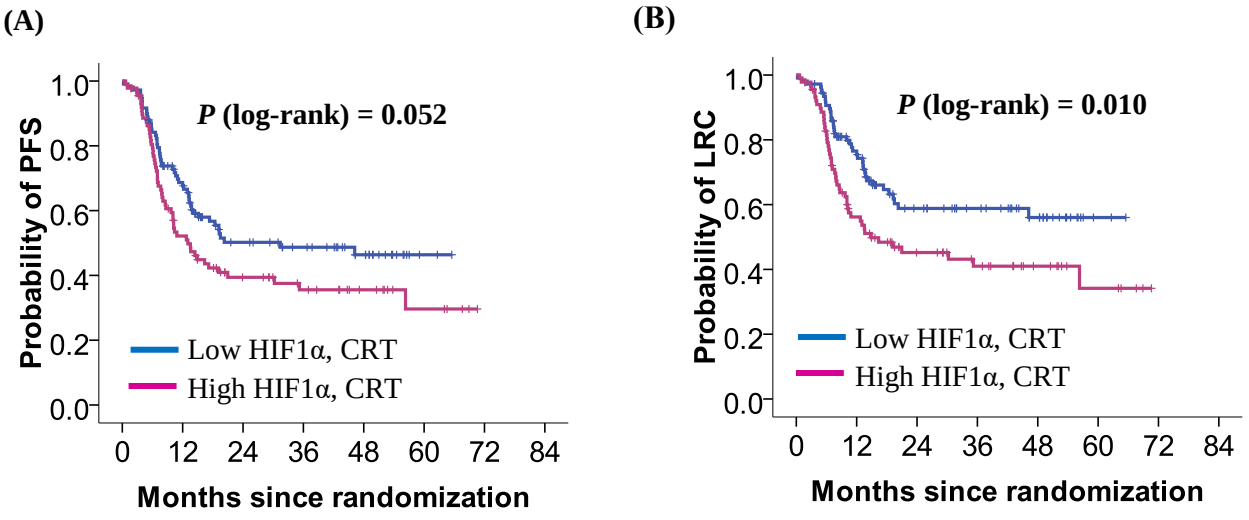
(*) Pearson's $\chi 2$ tests. Clinical stage is according to AJCC-UICC system ($8^{th}$ edition).

### 4.7. Prognostic significance of different biomarkers and patients' baseline parameters

As per the definition of prognostic biomarker proposed by Clark et al, the prognostic significance of each biomarker was analyzed in the CRT group which served as the control group (27).

#### 4.7.1. Prognostic significance of nuclear HIF1α expression

Among biomarkers, unadjusted analyses of HIF1α protein expression categorized at the median cut-point showed that the patients with tumors expressing lower levels of HIF1α had significantly longer LRC [HR (95%CI) =0.58 (0.38-0.89), 56.0 vs 41.0 months] as well as longer OS [HR (95%CI) =0.62 (0.42-0.91), 50.2 vs 31.7 months, Figure 16A-B ]. Patients with low HIF1α also showed a trend towards better PFS [HR (95%CI) =0.69 (0.47-1.01), 46.4 vs 35.6 months, Figure 16C] compared to the patients with tumors expressing high HIF1α. We also carried out univariate Cox regression analysis at different HIF1α HScore cutpoints. We observed that the low expression of HIF1α was numerically associated with improved PFS, LRC, and OS in the CRT group (Table 13).



**(C)**

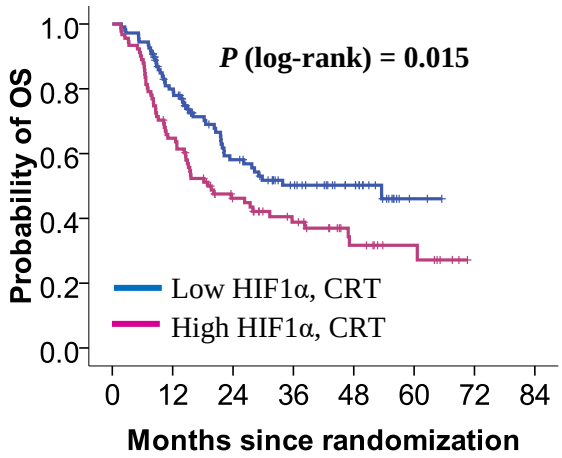



***Figure 16: Prognostic significance of HIF1α expression status in HNSCCs***

*Kaplan–Meier plots showing PFS (A), LRC (B), OS (C) according to HIF1α expression status in CRT group; HR=hazard ratio; CI=confidence interval.*

***Table 13: Cutpoint analysis to assess the prognostic role of HIF1α HScore***

| CRT (n=199) | | | PFS | | LRC | | OS | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low** | **High** | **HR (95% CI)** | ***P** value** | **HR (95% CI)** | ***P** value** | **HR (95% CI)** | ***P** value** |
| ≤10 &>10 | 28 | 171 | 0.75 (0.41-1.37) | 0.348 | 0.48 (0.22-1.03) | 0.06 | 0.51 (0.25-1.05) | 0.069 |
| ≤30 &>30 | 47 | 152 | 0.5 (0.28-0.88) | 0.015 | 0.52 (0.29-0.94) | 0.029 | 0.50 (0.28-0.88) | 0.015 |
| ≤50 &>50 | 71 | 128 | 0.69 (0.45-1.05) | 0.086 | 0.53 (0.33-0.87) | 0.011 | 0.74 (0.48-1.13) | 0.164 |
| ≤70 &>70 | 82 | 117 | 0.74 (0.5-1.11) | 0.143 | 0.63 (0.40-0.99) | 0.045 | 0.8 (0.54-1.2) | 0.281 |
| ≤90 &>90 | 108 | 91 | 0.69 (0.47-1.01) | 0.053 | 0.58 (0.38-0.89) | 0.011 | 0.62 (0.42-0.91) | 0.016 |
| ≤120 &>120 | 120 | 79 | 0.79 (0.54-1.16) | 0.231 | 0.72 (0.47-1.1) | 0.13 | 0.72 (0.49-1.05) | 0.089 |
| ≤160 &>160 | 138 | 61 | 0.74 (0.5-1.12) | 0.153 | 0.67 (0.43-1.04) | 0.073 | 0.62 (0.42-0.93) | 0.02 |
| ≤180 &>180 | 155 | 44 | 0.74 (0.47-1.17) | 0.197 | 0.64 (0.4-1.04) | 0.07 | 0.66 (0.43-1.02) | 0.062 |
| ≤240 &>240 | 169 | 30 | 0.59 (0.36-0.96) | 0.035 | 0.48 (0.29-0.82) | 0.006 | 0.51 (0.32-0.82) | 0.005 |

*Univariate Cox regression analysis. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval; PFS=progression free survival; LRC=loco-regional control; OS=overall survival

### 4.7.2. Prognostic significance of different EGFR based biomarkers

At the median HScore EGFR expression at membrane did not show any significant association with PFS [HR (95%CI)= 0.84 (0.58-1.23), 44.2 vs 40.0 months], LRC [HR (95%CI)= 0.79 (0.52-1.20), 54.0 vs 45.1 months] or OS [HR (95%CI)= 0.83 (0.57-1.22), 44.8 vs 40.7 months]. Similarly, EGFR expression at cytoplasm was not associated with any of the clinical endpoint [PFS: HR (95%CI)= 0.82 (0.56-1.20), 45.9 vs 35.7 months]; LRC: HR (95%CI)= 0.90 (0.59-1.37), 52.6 vs 44.1 months; OS: HR (95%CI)= 1.03 (0.69-1.52), 40.8 vs 45.9 months]. Univariate Cox analysis at different EGFR HScore cutpoints also did not show any significant association with clinical outcomes at any of the cutpoint (Table 14).

***Table 14A: Cutpoint analysis to assess the prognostic role of membrane EGFR HScore***

| CRT (n=206) | | | PFS | | LRC | | OS | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low (n)** | **High (n)** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** |
| ≤10 & >10 | 35 | 171 | 1.18 (0.73-1.92) | 0.503 | 1.19 (0.70-2.01) | 0.529 | 0.98 (0.59-1.63) | 0.947 |
| ≤20 & >20 | 46 | 160 | 0.95 (0.60-1.49) | 0.817 | 1.01 (0.62-1.64) | 0.983 | 0.84 (0.52-1.35) | 0.466 |
| ≤40 & >40 | 59 | 147 | 0.93 (0.61-1.41) | 0.727 | 0.93 (0.59-1.47) | 0.758 | 0.91 (0.60-1.38) | 0.65 |
| ≤60 & >60 | 73 | 133 | 0.87 (0.59-1.30) | 0.499 | 0.87 (0.56-1.35) | 0.541 | 0.86 (0.57-1.28) | 0.445 |
| ≤80 & >80 | 85 | 121 | 0.87 (0.59-1.28) | 0.488 | 0.84 (0.55-1.28) | 0.411 | 0.92 (0.62-1.35) | 0.667 |
| ≤100 & >100 | 103 | 103 | 0.84 (0.58-1.23) | 0.37 | 0.79 (0.52-1.20) | 0.264 | 0.83 (0.57-1.22) | 0.348 |
| ≤120 & >120 | 119 | 87 | 0.90 (0.61-1.32) | 0.584 | 0.92 (0.60-1.40) | 0.687 | 0.96 (0.65-1.41) | 0.816 |
| ≤140 & >140 | 141 | 65 | 0.87 (0.58-1.30) | 0.492 | 0.99 (0.63-1.56) | 0.973 | 0.90 (0.60-1.36) | 0.621 |
| ≤160 & >160 | 158 | 48 | 0.90 (0.57-1.41) | 0.642 | 1.12 (0.67-1.88) | 0.67 | 1.02 (0.64-1.63) | 0.927 |
| ≤200 & >200 | 177 | 29 | 0.89 (0.52-1.51) | 0.656 | 1.16 (0.62-2.19) | 0.644 | 1.04 (0.60-1.79) | 0.90 |
| ≤240 & >240 | 186 | 20 | 0.61 (0.34-1.09) | 0.094 | 0.82 (0.41-1.64) | 0.576 | 0.83 (0.44-1.55) | 0.557 |

***Table 14B: Cutpoint analysis to assess the prognostic role of cytoplasmic EGFR HScore***

| CRT (n=206) | | | PFS | | LRC | | OS | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low (n)** | **High (n)** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** |
| ≤20 & >20 | 15 | 191 | 1.07 (0.54-2.12) | 0.845 | 1.35 (0.68-2.70) | 0.389 | 0.80 (0.37-1.72) | 0.561 |
| ≤40 & >40 | 30 | 176 | 1.01 (0.60-1.72) | 0.966 | 1.31 (0.76-2.24) | 0.335 | 1.02 (0.60-1.73) | 0.957 |
| ≤60 & >60 | 47 | 159 | 0.90 (0.57-1.42) | 0.657 | 1.06 (0.66-1.72) | 0.808 | 0.82 (0.51-1.32) | 0.419 |
| ≤80 & >80 | 74 | 132 | 1.02 (0.69-1.50) | 0.938 | 1.19 (0.78-1.81) | 0.428 | 0.95 (0.64-1.41) | 0.797 |
| ≤100 & >100 | 83 | 123 | 0.99 (0.67-1.45) | 0.95 | 1.02 (0.67-1.55) | 0.931 | 0.97 (0.66-1.43) | 0.873 |
| ≤120 & >120 | 94 | 112 | 0.92 (0.63-1.35) | 0.674 | 0.99 (0.66-1.50) | 0.974 | 0.96 (0.65-1.40) | 0.816 |
| ≤140 & >140 | 121 | 85 | 0.82 (0.56-1.20) | 0.299 | 0.90 (0.59-1.37) | 0.626 | 1.03 (0.69-1.52) | 0.901 |
| ≤160 & >160 | 155 | 51 | 0.61 (0.40-0.91) | 0.017 | 0.68 (0.43-1.08) | 0.101 | 0.81 (0.53-1.26) | 0.352 |
| ≤180 & >180 | 190 | 16 | 1.13 (0.52-2.43) | 0.759 | 1.33 (0.54-3.27) | 0.541 | 1.38 (0.61-3.14) | 0.445 |
| ≤190 & >190 | 198 | 8 | 1.31 (0.41-4.13) | 0.647 | 1.68 (0.41-6.82) | 0.47 | 2.13 (0.53-8.64) | 0.289 |

*Univariate Cox regression analysis. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval; PFS=progression free survival; LRC=loco-regional control; OS=overall survival

Patients with tumor lacking pEGFRY1068 expression showed statistically significant better PFS compared to patients with tumor expressing pEGFRY1068 expression [HR (95%CI)= 0.63 (0.40-1.0), 44.8 vs 24.0 months, Figure 17], similar difference was not observed in LRC [HR (95%CI)= 0.88 (0.51-1.54), 50.3 vs 39.1 months] or OS [HR (95%CI)= 1.08 (0.64-1.84), 41.5 vs 36.7 months, Table 15].

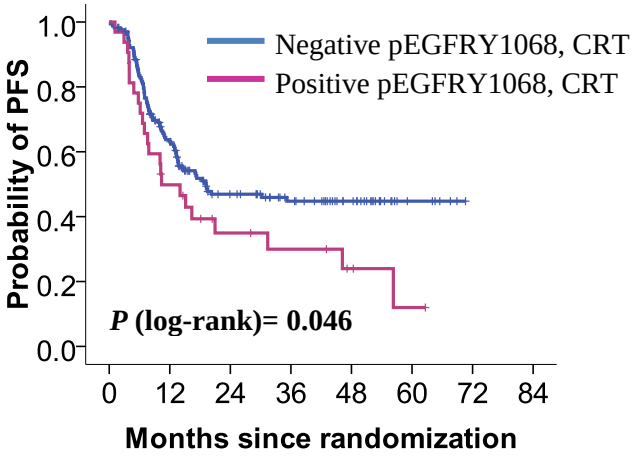



***Figure 17: Prognostic significance of pEGFRY1068 expression status in HNSCCs***

*Kaplan–Meier plots showing PFS according to pEGFRY1068 expression status in CRT group; HR=hazard ratio; CI=confidence interval.*

***Table 15: Univariate Cox analysis of biomarkers and clinical outcomes in the CRT group***

| **Variables** | **Progression free survival** | | | **Loco-regional control** | | | **Overall survival** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Events/n | HR (95% CI) | *P** | Events/n | HR (95% CI) | *P** | Events/n | HR (95% CI) | *P** |
| **pEGFRY1068** | | | | | | | | | |
| Negative | 83/168 | 0.63 (0.40-0.99) | **0.048** | 73/168 | 0.88 (0.51-1.54) | 0.655 | 89/168 | 1.08 (0.64-1.84) | 0.769 |
| Positive | 23/32 | | | 15/32 | | | 16/32 | | |
| **pEGFRY1173** | | | | | | | | | |
| Negative | 69/142 | 0.74 (0.48-1.14) | 0.17 | 61/142 | 0.93 (0.57-1.52) | 0.766 | 74/142 | 1.06 (0.67-1.70) | 0.801 |
| Positive | 29/46 | | | 21/46 | | | 23/46 | | |
| **EGFR FISH** | | | | | | | | | |
| Negative | 51/95 | 1.05 (0.66-1.69) | 0.827 | 40/95 | 0.94 (0.56-1.59) | 0.829 | 56/95 | 1.11 (0.70-1.76) | 0.651 |
| Positive | 26/48 | | | 22/48 | | | 27/48 | | |

Univariate Cox regression analysis. n=number of patients; CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval

pEGFRY1173 expression status did not show any association with any of the studied endpoints [PFS: HR (95%CI)= 0.74 (0.48-1.14), 45.5 vs 31.9 months; LRC: HR (95%CI)= 0.93 (0.57-1.52), 50.4 vs 45.8 months; OS: HR (95%CI)= 1.06 (0.67-1.70), 42.6 vs 38.6 months, Table 15]. We did not find any association between EGFR FISH status and clinical outcomes [PFS: HR (95%CI)= 1.05 (0.66-1.69), 42.2 vs 43.6 months; LRC: HR (95%CI)= 0.94 (0.56-1.59), 52.8 vs 50.3 months; OS: HR (95%CI)= 1.11 (0.70-1.76), 39.8 vs 37.6 months, Table 15].

### 4.7.3. Prognostic significance of different baseline parameters of the patients

In the CRT group, univariate Cox regression analysis of patient's different baseline characteristics showed that higher disease stage and oropharynx tumor site were significantly associated with poor PFS, LRC and OS (Table 16).

***Table 16: Univariate Cox analysis of baseline characteristics and clinical outcomes in the CRT group***

| **Variables** | **Progression free survival** | | | **Loco-regional control** | | | **Overall survival** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Events/n | HR (95% CI) | *P** | Events/n | HR (95% CI) | *P** | Events/n | HR (95% CI) | *P** |
| **Age** | | | | | | | | | |
| Below 60 | 82/148 | 1.46 (0.94-2.28) | 0.092 | 69/148 | 1.49 (0.91-2.43) | 0.111 | 83/148 | 1.59 (1.0-2.53) | **0.049** |
| Above 60 | 26/58 | | | 21/58 | | | 23/58 | | |
| **Sex** | | | | | | | | | |
| Male | 95/181 | 0.96 (0.54-1.71) | 0.884 | 79/181 | 0.95 (0.50-1.78) | 0.867 | 93/181 | 1.0 (0.56-1.79) | 1.0 |
| Female | 13/25 | | | 11/25 | | | 13/25 | | |
| **Disease stage**[#] | | | | | | | | | |
| III | 20/58 | 0.48 (0.30-0.78) | **0.003** | 15/58 | 0.43 (0.25-0.75) | **0.003** | 24/58 | 0.64 (0.40-1.00) | 0.051 |
| IV | 88/148 | | | 75/148 | | | 82/148 | | |
| **Tumor site** | | | | | | | | | |
| Oropharynx | 62/96 | 1.74 (1.19-2.56) | **0.004** | 50/96 | 1.58 (1.05-2.40) | **0.03** | 58/96 | 1.62 (1.10-2.37) | **0.014** |
| Others | 46/110 | | | 40/110 | | | 48/110 | | |
| Positive | 26/48 | | | 22/48 | | | 27/48 | | |
| High | 54/91 | | | 48/91 | | | 57/91 | | |

Univariate Cox regression analysis. n=number of patients; CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval

### 4.7.4. Multivariable Cox analysis to find independent prognostic biomarker

Multivariable analysis adjusted for confounding variables with a univariate *P*< 0.20 in Table 16 (age, clinical stage and site of tumor) identified HIF1α expression as an independent prognostic biomarker. Low expression of HIF1α remained significantly associated with PFS [HR (95%CI)=0.62 (0.42-0.93), *P*=0.020], LRC [HR (95%CI) =0.56 (0.37-0.86), *P*=0.007] and OS [HR (95%CI) =0.63 (0.43-0.93), *P*=0.019] in multivariable Cox regression analysis (Table 17).

***Table 17: Multivariable Cox analysis of baseline parameters and biomarkers in the CRT group***

| Variables | Univariate Cox analysis | | Multivariable Cox analysis* | |
|---|---|---|---|---|
| | **HR(95% CI)** | ***P* value** | **HR(95% CI)** | ***P* value** |
| **Progression-free survival (PFS)** | | | | |
| Age (below 60 vs 60 & above) | 1.46 (0.94-2.28) | 0.092 | 1.56 (0.97-2.52) | 0.066 |
| #Clinical stage (III vs IV) | 0.48 (0.30-0.78) | 0.003 | 0.41 (0.24-0.71) | **0.001** |
| Site of tumor (oropharynx vs others) | 1.74 (1.19-2.56) | 0.004 | - | - |
| pEGFRY1068 (negative vs positive) | 0.63 (0.40-1.0) | 0.048 | - | - |
| pEGFRY1173 (negative vs positive) | 0.74 (0.48-1.14) | 0.17 | - | - |
| HIF1α (low vs high) | 0.69 (0.47-1.01) | 0.053 | 0.62 (0.42-0.93) | **0.020** |
| **Loco-regional control (LRC)** | | | | |
| Age (below 60 vs 60 & above) | 1.49 (0.91-2.43) | 0.111 | 1.57 (0.96-2.56) | 0.075 |
| #Clinical stage (III vs IV) | 0.43 (0.25-0.75) | 0.003 | 0.39 (0.22-0.67) | **0.001** |
| Site of tumor (oropharynx vs others) | 1.58 (1.05-2.40) | 0.030 | - | - |
| HIF1α (low vs high) | 0.58 (0.38-0.89) | 0.011 | 0.56 (0.37-0.86) | **0.007** |
| **Overall survival (OS)** | | | | |
| Age (below 60 vs 60 & above) | 1.59 (1.0-2.53) | 0.049 | 1.65 (1.10-2.38) | **0.036** |
| #Clinical stage (III vs IV) | 0.64 (0.40-1.00) | 0.051 | - | - |
| Site of tumor (oropharynx vs others) | 1.62 (1.10-2.37) | 0.014 | 1.62 (1.10-2.38) | **0.015** |
| HIF1α (low vs high) | 0.62 (0.42-0.91) | 0.016 | 0.63 (0.43-0.93) | **0.019** |

* A multivariate Cox model using backward likelihood ratio method was applied to adjust for potential confounders (clinical characteristics associated with PFS, LRC or OS at *P*<0.20 in univariate analysis). HR=hazard ratio; CI=confidence interval. (-) data not available; #According to AJCC-UICC system (8th edition).

In addition, we also performed internal validation of the prognostic significance of HIF1α expression by bootstrap resampling method. Low HIF1α expression was significantly associated with better outcomes [PFS: HR (95%CI) =0.64 (0.43-0.96), *P*=0.031, c-index (95%CI)=0.61 (0.55-0.66); LRC: HR (95%CI) =0.58 (0.37-0.89), *P*=0.012, c-index (95%CI) =0.62 (0.56-0.68); OS: HR (95%CI) =0.63 (0.42-0.94), *P*=0.025, c-index (95%CI) =0.60 (0.54-0.65) in the CRT group.

### 4.8. Predictive significance of different biomarkers and patients' baseline parameters

#### 4.8.1. Predictive significance of different baseline parameters of the patients

In addition to different biomarkers, we also analyzed the predictive impact of baseline clinical and demographic characteristics. None of the studied clinical or demographic characteristics showed any predictive impact (Table 18).

***Table 18: Predictive impact of baseline clinical and demographic parameters of the patients***

| Variable | NCRT (n) | CRT (n) | Progression free survival | | | Loco-regional control | | | Overall survival | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | HR (95% CI) | *P** | *P*** | HR (95% CI) | *P** | *P*** | HR (95% CI) | *P** | *P*** |
| **Age (Years)** | | | | | | | | | | | |
| < 60 | 129 | 148 | 0.64 (0.45-0.90) | 0.01 | 0.42 | 0.64 (0.44-0.93) | 0.02 | 0.622 | 0.71 (0.51-0.99) | 0.044 | 0.246 |
| ≥ 60 | 69 | 58 | 0.83 (0.48-1.43) | 0.508 | | 0.77 (0.42-1.43) | 0.409 | | 1.05 (0.61-1.81) | 0.866 | |
| **Gender** | | | | | | | | | | | |
| Male | 171 | 181 | 0.70 (0.51-0.95) | 0.022 | 0.513 | 0.69 (0.49-0.96) | 0.03 | 0.469 | 0.79 (0.58-1.07) | 0.122 | 0.815 |
| Female | 27 | 25 | 0.53 (0.23-1.24) | 0.143 | | 0.49 (0.19-1.26) | 0.14 | | 0.73 (0.33-1.63) | 0.445 | |
| **Tumor site** | | | | | | | | | | | |
| Oropharynx | 100 | 96 | 0.60 (0.41-0.88) | 0.009 | 0.449 | 0.69 (0.45-1.04) | 0.075 | 0.707 | 0.73 (0.50-1.07) | 0.105 | 0.708 |
| Others | 98 | 108 | 0.78 (0.50-1.22) | 0.272 | | 0.62 (0.37-1.03) | 0.063 | | 0.84 (0.54-1.29) | 0.416 | |
| **Disease stage** | | | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| III | 40 | 58 | 0.47 (0.21-1.07) | 0.073 | 0.43 | 0.64 (0.27-1.51) | 0.31 | 0.98 | 0.52 (0.25-1.09) | 0.083 | 0.291 |
| IV | 158 | 148 | 0.67 (0.49-0.92) | 0.012 | | 0.62 (0.44-0.88) | 0.007 | | 0.80 (0.59-1.09) | 0.151 | |
| **Tobacco-alcohol habit profile** | | | | | | | | | | | |
| No habit | 16 | 14 | 0.92 (0.31-2.76) | 0.883 | 0.36 | 0.84 (0.27-2.62) | 0.767 | 0.318 | 1.04 (0.35-3.11) | 0.948 | 0.294 |
| Any one habit | 77 | 74 | 0.74 (0.46-1.17) | 0.192 | | 0.77 (0.46-1.30) | 0.329 | | 0.88 (0.57-1.37) | 0.575 | |
| Mix habits | 98 | 114 | 0.60 (0.40-0.91) | 0.017 | | 0.56 (0.35-0.88) | 0.013 | | 0.65 (0.43-0.99) | 0.043 | |
| *Univariate Cox regression analysis; ** interaction *P* value | | | | | | | | | | | |

### 4.8.2. Predictive significance of nuclear HIF1α expression

Predictive analysis at the median HScore cutpoint of 90 showed that in patients with a tumor expressing low HIF1α, no significant difference was seen in PFS, LRC, or OS with treatments in univariate Cox analysis (Figure 18). Interestingly, however, high expression of HIF1α in NCRT treated patients was found to be significantly associated with improved PFS [HR (95%CI) =0.55 (0.37-0.82), *P*=0.003], LRC [HR (95%CI) =0.55 (0.36-0.85), *P*=0.006] and OS [HR (95%CI) =0.54 (0.36-0.81), *P*=0.003] compared to patients expressing high HIF1α treated with CRT (Figure 18). However, A statistically significant qualitative interaction was observed between treatment effect and HIF1α expression status for OS [*P*=0.008] but not for PFS [*P*=0.137] or LRC [*P*=0.234].

The predictive significance of HIF1α expression was further validated by the bootstrap resampling method. Forest plots representing the interaction between treatments and HIF1α status for PFS, LRC, and OS are provided in Figure 19. Bootstrap resampling validation confirmed the predictive value of HIF1α for OS [*P* (interaction)= 0.007, c-index (95%CI) =0.57 (0.52-0.61)].

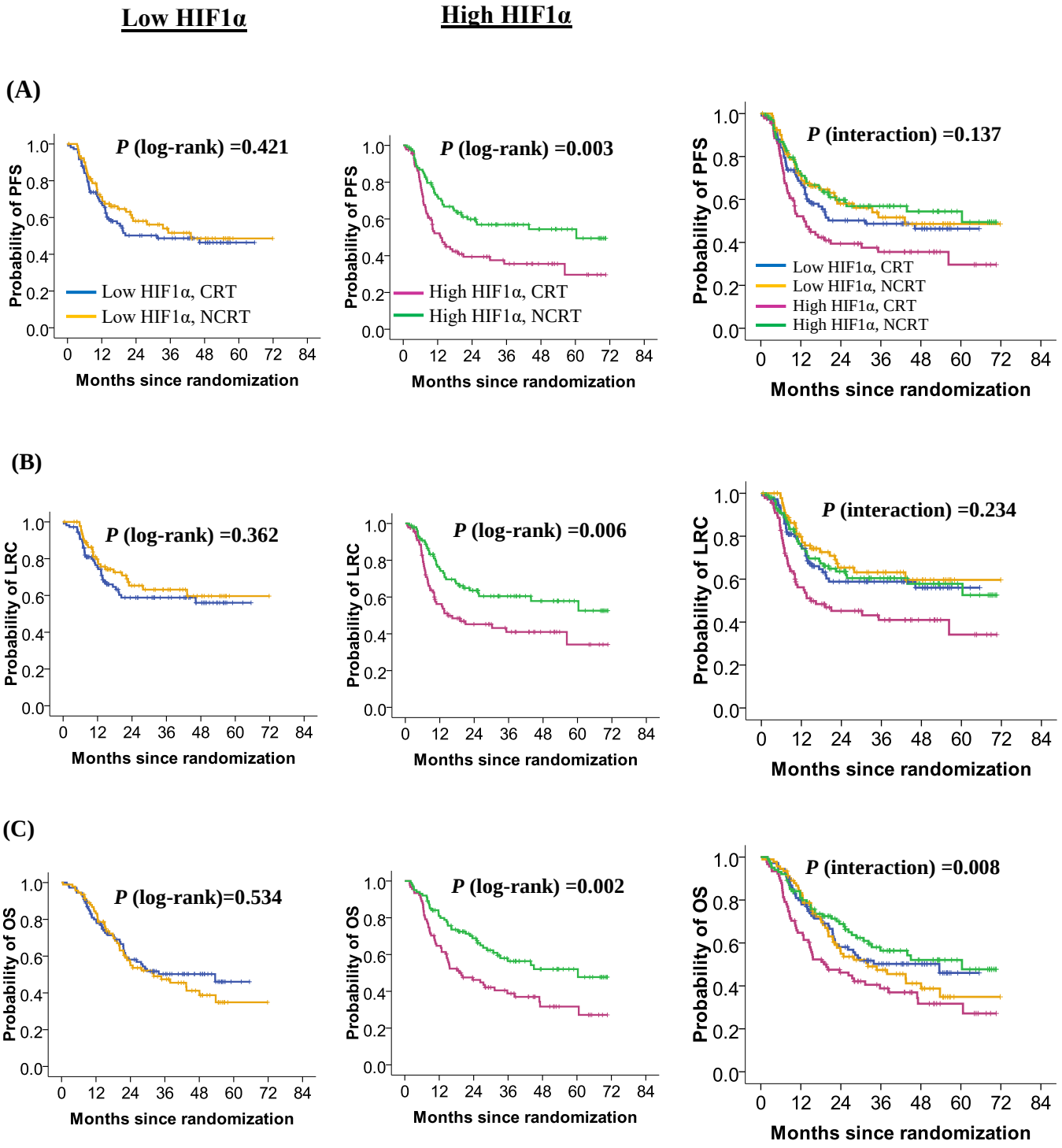



***Figure 18: HIF1α status showing qualitative interaction with treatment effect.*** *Kaplan–Meier plots showing, PFS (A), LRC (B) and OS (C) for LA-HNSCC patients according to HIF1α expression status and treatment group.*

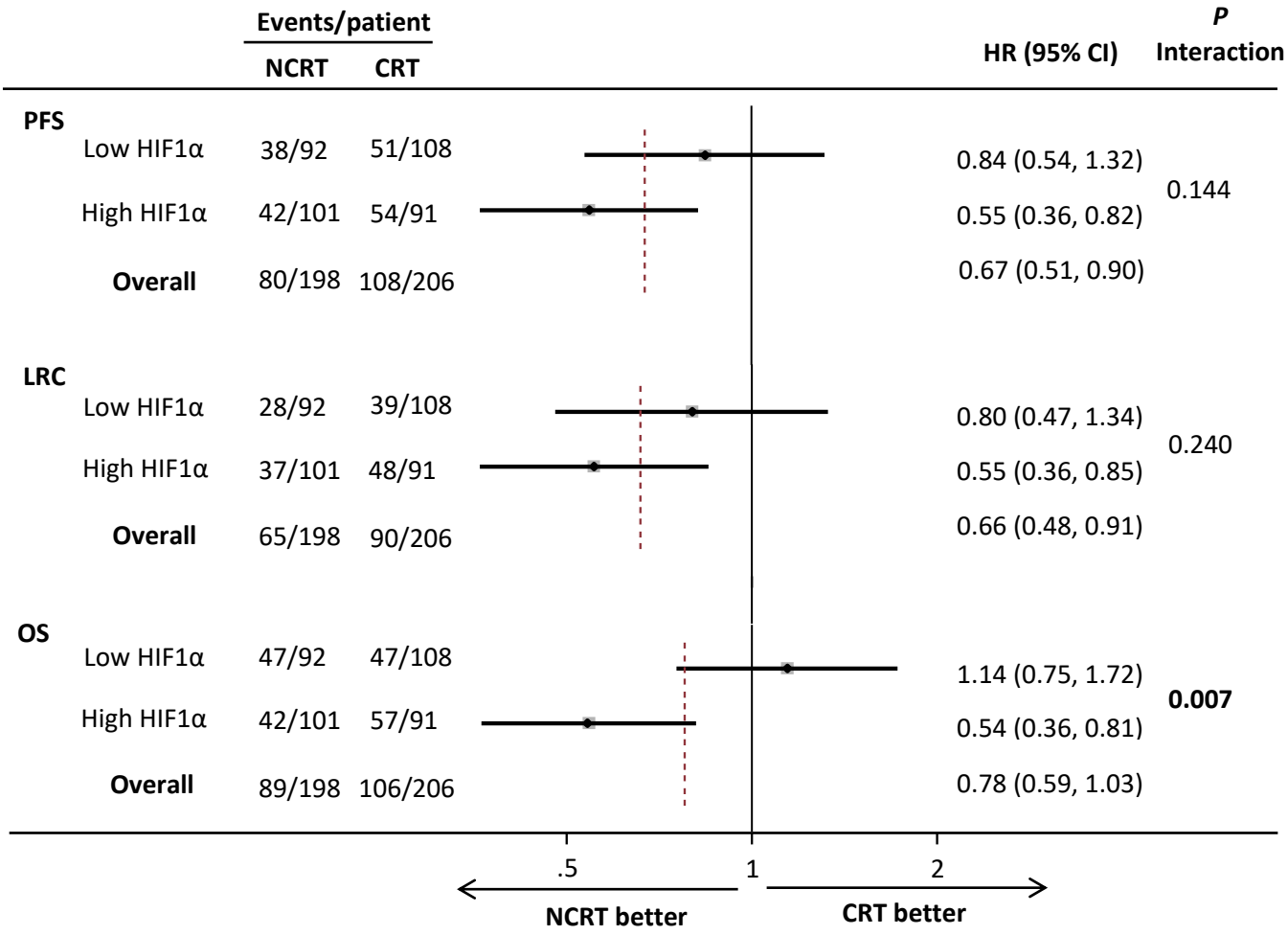



***Figure 19: Forest plot showing bootstrap resampling results for PFS, LRC and OS by HIF1α expression status.*** *A hazard ratio (HR) of less than 1 indicates a benefit with the addition of nimotuzumab; dotted line represents the respective hazard ratio for the overall study population.*

We also carried out the predictive analysis of HIF1α at different possible HScore cutpoints for PFS, LRC, and OS (Table 19). Overall, we observed that high expression of HIF1α was associated with better clinical outcomes in patients treated with NCRT compared to patients treated with CRT, with minimum interaction *P* value observed at the median cutpoint.

***Table 19: Cutpoint analysis to assess the predictive role of HIF1α HScore***

| Low HIF1α HScore | | | | | | High HIF1α HScore | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Progression free survival (PFS)** | | | | | | | | | | | | |
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year PFS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year PFS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | ***P* (Interaction)** |
| ≤10 | 23 | 28 | 45.8 vs 53.9 | 1.04 (0.44-2.47) | 0.92 | >10 | 170 | 171 | 52.8 vs 39.7 | 0.64 (0.47-0.87) | 0.005 | 0.311 |
| ≤30 | 36 | 47 | 57.2 vs 56.9 | 0.82 (0.40-1.69) | 0.588 | >30 | 157 | 152 | 51.0 vs 37.2 | 0.64 (0.47-0.88) | 0.006 | 0.579 |
| ≤50 | 51 | 71 | 56.7 vs 53.9 | 0.80 (0.45-1.44) | 0.454 | >50 | 142 | 128 | 50.6 vs 35.6 | 0.62 (0.44-0.87) | 0.005 | 0.498 |
| ≤70 | 73 | 82 | 54.4 vs 49.8 | 0.76 (0.46-1.25) | 0.277 | >70 | 120 | 117 | 50.5 vs 36.3 | 0.63 (0.44-0.91) | 0.012 | 0.558 |
| **≤90** | **92** | **108** | **48.6 vs 46.4** | **0.84 (0.55-1.28)** | **0.422** | **>90** | **101** | **91** | **54.4 vs 35.6** | **0.55 (0.37-0.82)** | **0.003** | **0.137** |
| ≤120 | 101 | 120 | 47.9 vs 43.1 | 0.79 (0.53-1.17) | 0.235 | >120 | 92 | 79 | 55.4 vs 39.0 | 0.57 (0.37-0.88) | 0.011 | 0.253 |
| ≤160 | 128 | 138 | 48.4 vs 44.1 | 0.79 (0.55-1.12) | 0.182 | >160 | 65 | 61 | 58.0 vs 35.0 | 0.51 (0.31-0.85) | 0.009 | 0.165 |
| ≤180 | 155 | 155 | 48.9 vs 42.7 | 0.76 (0.55-1.05) | 0.10 | >180 | 38 | 44 | 62.6 vs 36.8 | 0.47 (0.25-0.91) | 0.026 | 0.149 |
| ≤240 | 166 | 169 | 49.0 vs 43.9 | 0.79 (0.58-1.08) | 0.141 | >240 | 27 | 30 | 69.3 vs 25.8 | 0.28 (0.12-0.65) | 0.003 | 0.016 |
| **Loco-regional control (LRC)** | | | | | | | | | | | | |
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year LRC (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year LRC (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | ***P* (Interaction)** |
| ≤10 | 23 | 28 | 57.1 vs 69.9 | 1.04 (0.33-3.29) | 0.942 | >10 | 170 | 171 | 58.9 vs 46.1 | 0.63 (0.45-0.88) | 0.007 | 0.451 |
| ≤30 | 36 | 47 | 67.9 vs 68.0 | 0.70 (0.28-1.76) | 0.45 | >30 | 157 | 152 | 57.0 vs 43.9 | 0.64 (0.45-0.90) | 0.01 | 0.877 |
| ≤50 | 51 | 71 | 67.5 vs 64.8 | 0.68 (0.33-1.41) | 0.298 | >50 | 142 | 128 | 56.1 vs 41.6 | 0.62 (0.43-0.89) | 0.009 | 0.831 |
| ≤70 | 73 | 82 | 61.5 vs 58.7 | 0.73 (0.41-1.30) | 0.289 | >70 | 120 | 117 | 57.1 vs 43.0 | 0.62 (0.42-0.92) | 0.017 | 0.613 |
| **≤90** | **92** | **108** | **59.7 vs 56.0** | **0.80 (0.49-1.30)** | **0.363** | **>90** | **101** | **91** | **57.8 vs 41.0** | **0.55 (0.36-0.85)** | **0.006** | **0.234** |
| ≤120 | 101 | 120 | 59.7 vs 51.5 | 0.70 (0.44-1.10) | 0.122 | >120 | 92 | 79 | 57.6 vs 45.5 | 0.61 (0.39-0.96) | 0.034 | 0.633 |
| ≤160 | 128 | 138 | 57.6 vs 52.6 | 0.74 (0.50-1.11) | 0.15 | >160 | 65 | 61 | 60.2 vs 40.8 | 0.54 (0.31-0.92) | 0.022 | 0.315 |
| ≤180 | 155 | 155 | 56.8 vs 51.4 | 0.75 (0.52-1.08) | 0.126 | >180 | 38 | 44 | 64.8 vs 41.1 | 0.48 (0.24-0.95) | 0.035 | 0.181 |
| ≤240 | 166 | 169 | 56.8 vs 52.5 | 0.78 (0.55-1.11) | 0.168 | >240 | 27 | 30 | 69.3 vs 28.6 | 0.31 (0.13-0.72) | 0.006 | 0.024 |

***Table 19: Cutpoint analysis to assess the predictive role of HIF1α HScore (continued)***

**Overall survival (OS)**

| Low HIF1α HScore | | | | | | High HIF1α HScore | | | | | | ***P*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year OS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year OS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **(Interaction)** |
| ≤10 | 23 | 28 | 34.2 vs 64.4 | 2.26 (0.95-5.40) | 0.066 | >10 | 170 | 171 | 48.5 vs 38.4 | 0.68 (0.50-0.92) | 0.011 | 0.009 |
| ≤30 | 36 | 47 | 39.2 vs 61.7 | 1.75 (0.88-3.50) | 0.111 | >30 | 157 | 152 | 48.4 vs 36.5 | 0.65 (0.47-0.89) | 0.007 | 0.01 |
| ≤50 | 51 | 71 | 39.6 vs 48.6 | 1.18 (0.70-1.97) | 0.532 | >50 | 142 | 128 | 49.5 vs 37.7 | 0.65 (0.47-0.92) | 0.014 | 0.053 |
| ≤70 | 73 | 82 | 42.7 vs 44.8 | 1.01 (0.64-1.59) | 0.978 | >70 | 120 | 117 | 49.4 vs 39.0 | 0.66 (0.46-0.95) | 0.026 | 0.137 |
| **≤90** | **92** | **108** | **41.2 vs 50.2** | **1.14 (0.76-1.70)** | **0.534** | **>90** | **101** | **91** | **52.1 vs 31.7** | **0.54 (0.36-0.81)** | **0.003** | **0.008** |
| ≤120 | 101 | 120 | 40.0 vs 46.4 | 1.05 (0.72-1.53) | 0.801 | >120 | 92 | 79 | 53.9 vs 34.3 | 0.55 (0.36-0.84) | 0.006 | 0.02 |
| ≤160 | 128 | 138 | 44.8 vs 45.9 | 0.99 (0.70-1.39) | 0.93 | >160 | 65 | 61 | 51.1 vs 31.4 | 0.50 (0.30-0.80) | 0.004 | 0.017 |
| ≤180 | 155 | 155 | 45.1 vs 43.4 | 0.90 (0.65-1.23) | 0.5 | >180 | 38 | 44 | 54.1 vs 33.3 | 0.49 (0.26-0.90) | 0.022 | 0.069 |
| ≤240 | 166 | 169 | 45.7 vs 44.7 | 0.90 (0.66-1.23) | 0.517 | >240 | 27 | 30 | 54.6 vs 23.1 | 0.37 (0.18-0.76) | 0.007 | 0.017 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; NCRT=nimotuzumab plus cisplatin-radiation; HR=hazard ratio; CI=confidence interval.

### 4.8.3. Predictive significance of different EGFR based biomarkers

We next analyzed the predictive impact of the membrane and cytoplasmic EGFR based biomarkers dichotomized at median HScore cutpoint. Univariate Cox analysis showed that PFS [HR (95%CI) =0.61 (0.41-0.92), *P*=0.02] and LRC [HR (95%CI) =0.59 (0.38-0.92), *P*=0.021] were significantly improved in the patients expressing high membrane EGFR with NCRT versus CRT, while the difference in OS was not statistically significant [HR (95%CI) =0.69 (0.46-1.03), *P*=0.071]. Improvement in PFS, LRC or OS with NCRT versus CRT was not observed in patients with low membrane EGFR expression (Figure 20).

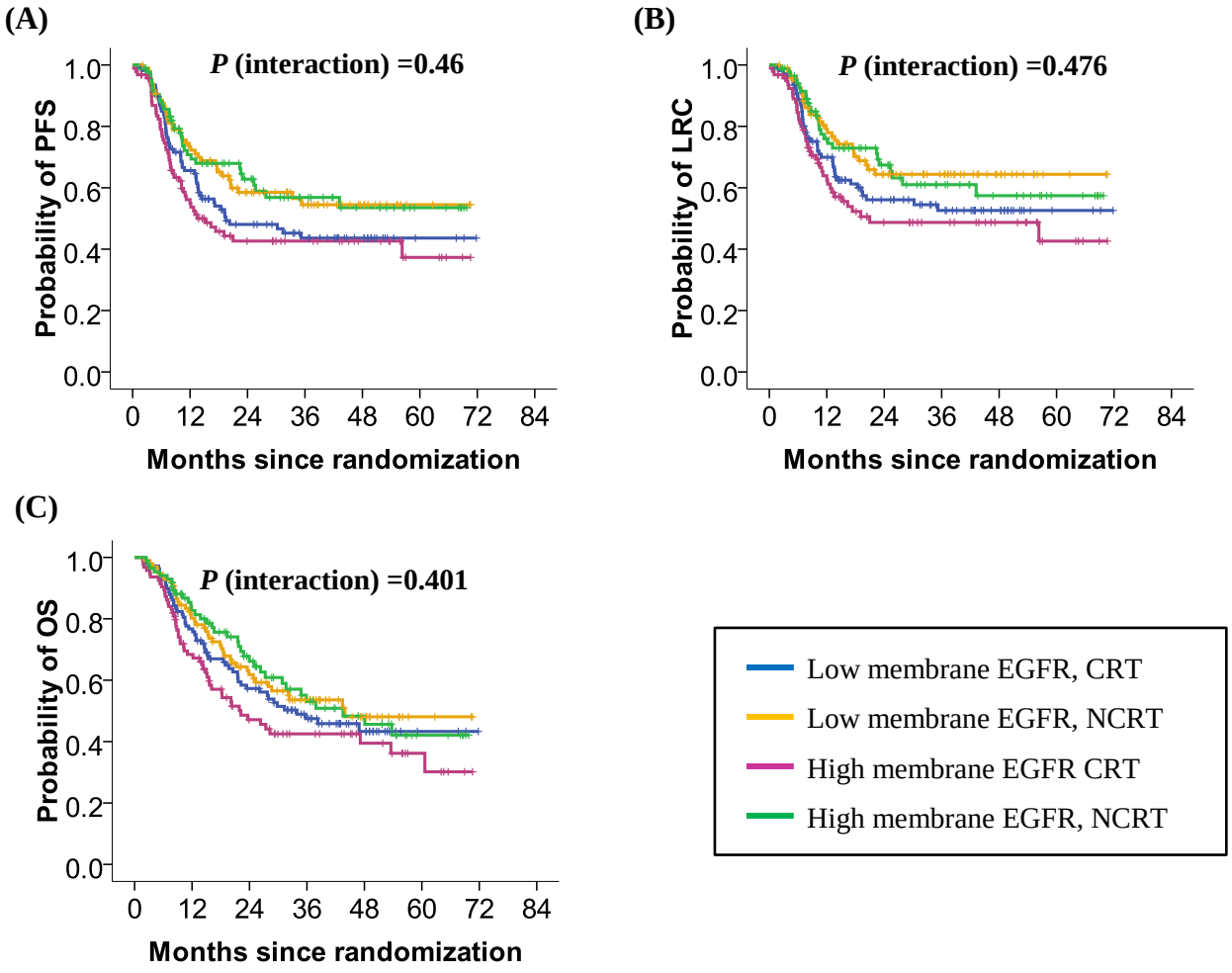



***Figure 20: Kaplan-Meier plots showing clinical outcomes according to the membrane EGFR status and treatment.*** *PFS (A), LRC (B) and (C) OS.*

Similar associations were also observed between cytoplasmic EGFR status and treatment effect. Patients with high cytoplasmic EGFR expression had statistically significant better PFS [HR (95%CI) =0.58 (0.37-0.90), *P*=0.016] and LRC [HR (95%CI) =0.51 (0.31-0.85), *P*=0.01] but not OS [HR (95%CI) =0.76 (0.49-1.18), *P*=0.228] with NCRT versus CRT (Figure 21). Also, we did not find any significant interaction between EGFR expression status and treatment effect at any of the studied cut-points (Table 20-21).

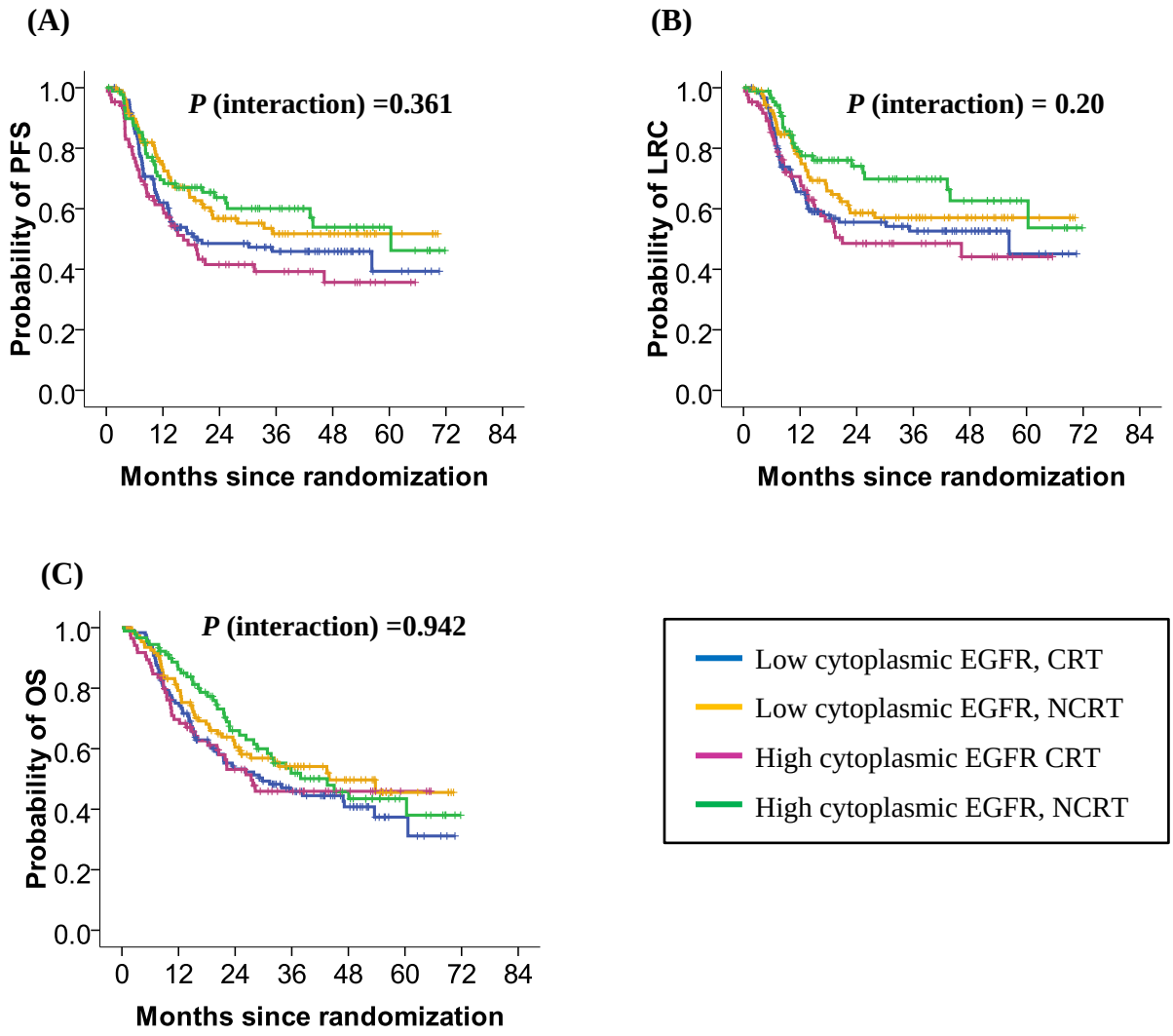



***Figure 21: Kaplan-Meier plots showing clinical outcomes according to the cytoplasmic EGFR status and treatment.*** *PFS (A), LRC (B) and (C) OS.*

***Table 20: Cutpoint analysis to assess the predictive role of membrane EGFR HScore***

| Low EGFR HScore | | | | | | High EGFR HScore | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Progression free survival (PFS)** | | | | | | | | | | | | |
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year PFS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year PFS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | ***P* (Interaction)** |
| ≤10 | 29 | 35 | 63.2 vs 35.6 | 0.45 (0.21-0.95) | 0.037 | >10 | 169 | 171 | 50.6 vs 43.3 | 0.73 (0.53-1.0) | 0.047 | 0.256 |
| ≤40 | 53 | 59 | 62.2 vs 42.4 | 0.54 (0.30-0.97) | 0.037 | >40 | 145 | 147 | 48.6 vs 42.1 | 0.73 (0.52-1.02) | 0.064 | 0.408 |
| ≤60 | 74 | 73 | 59.4 vs 44.9 | 0.62 (0.37-1.01) | 0.055 | >60 | 124 | 133 | 48.4 vs 40.6 | 0.72 (0.50-1.02) | 0.065 | 0.654 |
| ≤80 | 92 | 85 | 54.0 vs 43.8 | 0.73 (0.47-1.14) | 0.165 | >80 | 106 | 121 | 51.8 vs 41.0 | 0.64 (0.43-0.94) | 0.022 | 0.591 |
| **≤100** | **101** | **103** | **53.3 vs 44.2** | **0.75 (0.50-1.12)** | **0.156** | **>100** | **97** | **103** | **52.7 vs 40.0** | **0.61 (0.41-0.92)** | **0.02** | **0.46** |
| ≤120 | 120 | 119 | 52.2 vs 42.2 | 0.72 (0.50-1.05) | 0.085 | >120 | 78 | 87 | 54.8 vs 42.0 | 0.61 (0.39-0.97) | 0.038 | 0.537 |
| ≤140 | 133 | 141 | 50.7 vs 42.9 | 0.74 (0.52-1.05) | 0.094 | >140 | 65 | 65 | 58.2 vs 40.2 | 0.55 (0.33-0.93) | 0.025 | 0.294 |
| ≤160 | 156 | 158 | 51.7 vs 42.8 | 0.71 (0.52-0.99) | 0.041 | >160 | 42 | 48 | 56.5 vs 38.4 | 0.56 (0.29-1.06) | 0.072 | 0.435 |
| ≤180 | 166 | 168 | 51.5 vs 42.6 | 0.68 (0.50-0.94) | 0.019 | >180 | 32 | 38 | 57.0 vs 38.9 | 0.65 (0.32-1.32) | 0.236 | 0.804 |
| ≤200 | 171 | 177 | 51.1 vs 43.2 | 0.69 (0.50-0.94) | 0.018 | >200 | 27 | 29 | 60.8 vs 36.6 | 0.63 (0.29-1.37) | 0.245 | 0.786 |
| ≤240 | 175 | 186 | 51.1 vs 44.5 | 0.72 (0.53-0.97) | 0.034 | >240 | 23 | 20 | 62.4 vs 22.3 | 0.43 (0.18-1.01) | 0.053 | 0.204 |
| **Loco-regional control (LRC)** | | | | | | | | | | | | |
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year LRC (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year LRC (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | ***P* (Interaction)** |
| ≤10 | 29 | 35 | 71.1 vs 43.9 | 0.37 (0.15-0.89) | 0.026 | >10 | 169 | 171 | 57.2 vs 50.5 | 0.73 (0.52-1.03) | 0.070 | 0.16 |
| ≤40 | 53 | 59 | 73.3 vs 51.3 | 0.43 (0.22-0.86) | 0.016 | >40 | 145 | 147 | 53.9 vs 48.8 | 0.75 (0.52-1.08) | 0.120 | 0.17 |
| ≤60 | 74 | 73 | 69.5 vs 53.1 | 0.55 (0.32-0.97) | 0.039 | >60 | 124 | 133 | 52.9 vs 47.5 | 0.73 (0.49-1.07) | 0.108 | 0.43 |
| ≤80 | 92 | 85 | 64.0 vs 53.6 | 0.70 (0.43-1.15) | 0.16 | >80 | 106 | 121 | 55.6 vs 46.6 | 0.63 (0.42-0.97) | 0.034 | 0.75 |
| **≤100** | **101** | **103** | **62.6 vs 54.0** | **0.74 (0.47-1.17)** | **0.193** | **>100** | **97** | **103** | **57.0 vs 45.1** | **0.59 (0.38-0.92)** | **0.021** | **0.48** |
| ≤120 | 120 | 119 | 60.9 vs 50.4 | 0.69 (0.46-1.04) | 0.078 | >120 | 78 | 87 | 59.1 vs 48.4 | 0.62 (0.37-1.02) | 0.060 | 0.70 |
| ≤140 | 133 | 141 | 58.8 vs 49.9 | 0.71 (0.48-1.03) | 0.073 | >140 | 65 | 65 | 63.7 vs 48.8 | 0.56 (0.31-1.01) | 0.054 | 0.49 |
| ≤160 | 156 | 158 | 59.8 vs 49.7 | 0.65 (0.46-0.93) | 0.019 | >160 | 42 | 48 | 59.5 vs 48.2 | 0.69 (0.34-1.41) | 0.306 | 0.93 |
| ≤180 | 166 | 168 | 59.0 vs 49.5 | 0.63 (0.45-0.90) | 0.01 | >180 | 32 | 38 | 60.9 vs 49.7 | 0.84 (0.38-1.87) | 0.666 | 0.57 |
| ≤200 | 171 | 177 | 58.6 vs 50.3 | 0.63 (0.45-0.89) | 0.009 | >200 | 27 | 29 | 63.3 vs 47.8 | 0.89 (0.38-2.11) | 0.798 | 0.51 |
| ≤240 | 175 | 186 | 58.3 vs 51.5 | 0.67 (0.48-0.94) | 0.02 | >240 | 23 | 20 | 56.3 vs 32.2 | 0.60 (0.23-1.56) | 0.294 | 0.75 |

***Table 20: Cutpoint analysis to assess the predictive role of membrane EGFR HScore (continued)***

**Overall survival (OS)**

| Low EGFR (membrane) HScore | | | | | | High EGFR (membrane) HScore | | | | | | ***P*** **(Interaction)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year OS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year OS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | |
| ≤10 | 29 | 35 | 56.1 vs 45.9 | 0.68 (0.33-1.41) | 0.3 | >10 | 169 | 171 | 46.0 vs 41.8 | 0.81 (0.59-1.09) | 0.164 | 0.59 |
| ≤40 | 53 | 59 | 55.4 vs 41.9 | 0.73 (0.42-1.26) | 0.264 | >40 | 145 | 147 | 44.5 vs 43.3 | 0.80 (0.58-1.12) | 0.196 | 0.72 |
| ≤60 | 74 | 73 | 53.0 vs 44.7 | 0.74 (0.45-1.19) | 0.21 | >60 | 124 | 133 | 44.2 vs 41.8 | 0.81 (0.57-1.15) | 0.244 | 0.71 |
| ≤80 | 92 | 85 | 49.7 vs 43.6 | 0.80 (0.518-1.2) | 0.295 | >80 | 106 | 121 | 45.7 vs 42.4 | 0.77 (0.53-1.13) | 0.184 | 0.97 |
| **≤100** | **101** | **103** | **47.3 vs 44.8** | **0.89 (0.59-1.32)** | **0.548** | **>100** | **97** | **103** | **48.2 vs 40.7** | **0.69 (0.46-1.03)** | **0.071** | **0.40** |
| ≤120 | 120 | 119 | 45.4 vs 41.0 | 0.87 (0.61-1.25) | 0.456 | >120 | 78 | 87 | 52.4 vs 44.4 | 0.65 (0.41-1.03) | 0.069 | 0.32 |
| ≤140 | 133 | 141 | 44.4 vs 42.8 | 0.90 (0.64-1.26) | 0.535 | >140 | 65 | 65 | 55.4 vs 41.4 | 0.57 (0.34-0.96) | 0.034 | 0.14 |
| ≤160 | 156 | 158 | 47.0 vs 42.3 | 0.80 (0.58-1.09) | 0.158 | >160 | 42 | 48 | 51.8 vs 42.8 | 0.72 (0.39-1.33) | 0.295 | 0.77 |
| ≤180 | 166 | 168 | 47.0 vs 42.3 | 0.79 (0.58-1.07) | 0.124 | >180 | 32 | 38 | 52.6 vs 42.7 | 0.74 (0.36-1.51) | 0.409 | 0.89 |
| ≤200 | 171 | 177 | 47.1 vs 42.9 | 0.80 (0.59-1.08) | 0.138 | >200 | 27 | 29 | 52.6 vs 46.4 | 0.68 (0.31-1.49) | 0.335 | 0.72 |
| ≤240 | 175 | 186 | 47.3 vs 42.6 | 0.81 (0.60-1.09) | 0.166 | >240 | 23 | 20 | 51.9 vs 37.5 | 0.57 (0.24-1.35) | 0.199 | 0.44 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; NCRT=nimotuzumab plus cisplatin-radiation; HR=hazard ratio; CI=confidence interval.

***Table 21: Cutpoint analysis to assess the predictive role of cytoplasmic EGFR HScore***

| Low EGFR HScore | | | | | | High EGFR HScore | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Progression free survival (PFS)** | | | | | | | | | | | | |
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year PFS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year PFS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | ***P* (Interaction)** |
| ≤20 | 14 | 15 | 76.9 vs 40.0 | 0.77 (0.26-2.28) | 0.63 | >20 | 184 | 191 | 50.4 vs 42.0 | 0.78 (0.59-1.05) | 0.102 | 0.902 |
| ≤40 | 28 | 30 | 60.4 vs 40.2 | 0.72 (0.34-1.52) | 0.389 | >40 | 170 | 176 | 51.2 vs 42.3 | 0.80 (0.59-1.08) | 0.14 | 0.715 |
| ≤60 | 41 | 47 | 56.1 vs 43.6 | 0.89 (0.48-1.67) | 0.725 | >60 | 157 | 159 | 51.8 vs 41.6 | 0.75 (0.55-1.03) | 0.078 | 0.688 |
| ≤80 | 67 | 74 | 51.4 vs 41.3 | 0.84 (0.52-1.34) | 0.46 | >80 | 131 | 132 | 53.5 vs 42.1 | 0.74 (0.53-1.06) | 0.097 | 0.706 |
| ≤100 | 77 | 83 | 54.8 vs 43.0 | 0.77 (0.49-1.21) | 0.253 | >100 | 121 | 123 | 51.3 vs 41.0 | 0.78 (0.54-1.11) | 0.166 | 0.971 |
| ≤120 | 91 | 94 | 55.2 vs 44.4 | 0.74 (0.49-1.13) | 0.166 | >120 | 107 | 112 | 50.3 vs 39.9 | 0.80 (0.55-1.17) | 0.257 | 0.765 |
| **≤140** | **108** | **121** | **51.7 vs 45.9** | **0.79 (0.54-1.14)** | **0.204** | **>140** | **90** | **85** | **53.9 vs 35.7** | **0.76 (0.49-1.18)** | **0.228** | **0.942** |
| ≤160 | 138 | 155 | 52.3 vs 46.9 | 0.82 (0.59-1.15) | 0.254 | >160 | 60 | 51 | 53.4 vs 27.4 | 0.65 (0.39-1.10) | 0.111 | 0.477 |
| ≤180 | 167 | 190 | 50.8 vs 41.2 | 0.75 (0.56-1.02) | 0.066 | >180 | 31 | 16 | 64.0 vs 51.4 | 1.12 (0.43-2.90) | 0.812 | 0.44 |
| **Loco-regional control (LRC)** | | | | | | | | | | | | |
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year LRC (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year LRC (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | ***P* (Interaction)** |
| ≤20 | 14 | 15 | 76.9 vs 40.0 | 0.26 (0.07-0.97) | 0.44 | >20 | 184 | 191 | 57.7 vs 50.2 | 0.71 (0.51-0.99) | 0.041 | 0.146 |
| ≤40 | 28 | 30 | 71.4 vs 40.2 | 0.36 (0.15-0.87) | 0.023 | >40 | 170 | 176 | 57.3 vs 51.1 | 0.73 (0.52-1.03) | 0.07 | 0.147 |
| ≤60 | 41 | 47 | 63.7 vs 48.4 | 0.58 (0.29-1.15) | 0.121 | >60 | 157 | 159 | 58.4 vs 49.8 | 0.68 (0.48-0.98) | 0.039 | 0.68 |
| ≤80 | 67 | 74 | 56.1 vs 47.4 | 0.69 (0.42-1.15) | 0.154 | >80 | 131 | 132 | 61.5 vs 50.4 | 0.64 (0.42-0.96) | 0.033 | 0.792 |
| ≤100 | 77 | 83 | 59.0 vs 51.0 | 0.71 (0.43-1.16) | 0.166 | >100 | 121 | 123 | 59.9 vs 48.2 | 0.62 (0.41-0.95) | 0.028 | 0.707 |
| ≤120 | 91 | 94 | 58.5 vs 51.4 | 0.75 (0.47-1.17) | 0.204 | >120 | 107 | 112 | 60.0 vs 47.8 | 0.58 (0.37-0.92) | 0.019 | 0.45 |
| **≤140** | **108** | **121** | **57.0 vs 52.6** | **0.79 (0.52-1.18)** | **0.248** | **>140** | **90** | **85** | **62.6 vs 44.1** | **0.51 (0.31-0.85)** | **0.01** | **0.2** |
| ≤160 | 138 | 155 | 58.4 vs 54.1 | 0.77 (0.53-1.13) | 0.18 | >160 | 60 | 51 | 62.3 vs 34.4 | 0.40 (0.22-0.75) | 0.004 | 0.088 |
| ≤180 | 167 | 190 | 57.8 vs 48.5 | 0.66 (0.48-0.93) | 0.017 | >180 | 31 | 16 | 69.9 vs 61.2 | 0.68 (0.22-2.08) | 0.498 | 0.93 |

***Table 21: Cutpoint analysis to assess the predictive role of cytoplasmic EGFR HScore (continued)***

**Overall survival (OS)**

| Low EGFR HScore | | | | | | High EGFR HScore | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year OS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | **Cut point** | **NCRT (n)** | **CRT (n)** | **4 year OS (months)** | **HR (95% CI) (NCRT vs CRT)** | ***P**** | ***P* (Interaction)** |
| ≤20 | 14 | 15 | 57.1 vs 52.5 | 0.77 (0.26-2.28) | 0.63 | >20 | 184 | 191 | 46.5 vs 41.4 | 0.78 (0.59-1.05) | 0.102 | 0.902 |
| ≤40 | 28 | 30 | 55.2 vs 43.2 | 0.72 (0.34-1.52) | 0.389 | >40 | 170 | 176 | 46.1 vs 42.4 | 0.80 (0.59-1.08) | 0.14 | 0.715 |
| ≤60 | 41 | 47 | 52.0 vs 50.1 | 0.89 (0.48-1.67) | 0.725 | >60 | 157 | 159 | 46.6 vs 40.4 | 0.75 (0.55-1.03) | 0.078 | 0.688 |
| ≤80 | 67 | 74 | 50.6 vs 43.4 | 0.84 (0.52-1.35) | 0.46 | >80 | 131 | 132 | 46.8 vs 41.6 | 0.74 (0.53-1.06) | 0.097 | 0.706 |
| ≤100 | 77 | 83 | 51.8 vs 47.4 | 0.77 (0.49-1.21) | 0.253 | >100 | 121 | 123 | 45.6 vs 40.5 | 0.78 (0.54-1.11) | 0.166 | 0.971 |
| ≤120 | 91 | 94 | 46.9 vs 40.5 | 0.74 (0.49-1.13) | 0.166 | >120 | 107 | 112 | 52.4 vs 43.9 | 0.80 (0.55-1.17) | 0.257 | 0.765 |
| **≤140** | **108** | **121** | **49.7 vs 40.8** | **0.79 (0.54-1.14)** | **0.204** | **>140** | **90** | **85** | **45.7 vs 45.9** | **0.76 (0.49-1.18)** | **0.228** | **0.942** |
| ≤160 | 138 | 155 | 50.4 vs 43.5 | 0.82 (0.59-1.15) | 0.254 | >160 | 60 | 51 | 43.0 vs 38.5 | 0.65 (0.39-1.10) | 0.111 | 0.477 |
| ≤180 | 167 | 190 | 50.0 vs 41.4 | 0.75 (0.56-1.02) | 0.066 | >180 | 31 | 16 | 35.2 vs 55.1 | 1.12 (0.43-2.90) | 0.812 | 0.44 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; NCRT=nimotuzumab plus cisplatin-radiation; HR=hazard ratio; CI=confidence interval.

Further, patients with tumors lacking expression of pEGFRY1068 had significantly improved clinical outcomes with NCRT compared to CRT [PFS: HR (95%CI)= 0.66 (0.48-0.92), *P*=0.014; LRC: HR (95%CI) =0.63 (0.44-0.90), *P*=0.012; OS: HR (95%CI)= 0.71 (0.52-0.96), *P*=0.029]. No similar significant differences in the clinical outcomes were seen with the treatments in patients with a tumor expressing pEGFRY1068 (Figure 22). We did not find any interaction between treatment effect and pEGFRY1068 status for PFS, LRC, or OS (Figure 25-27).

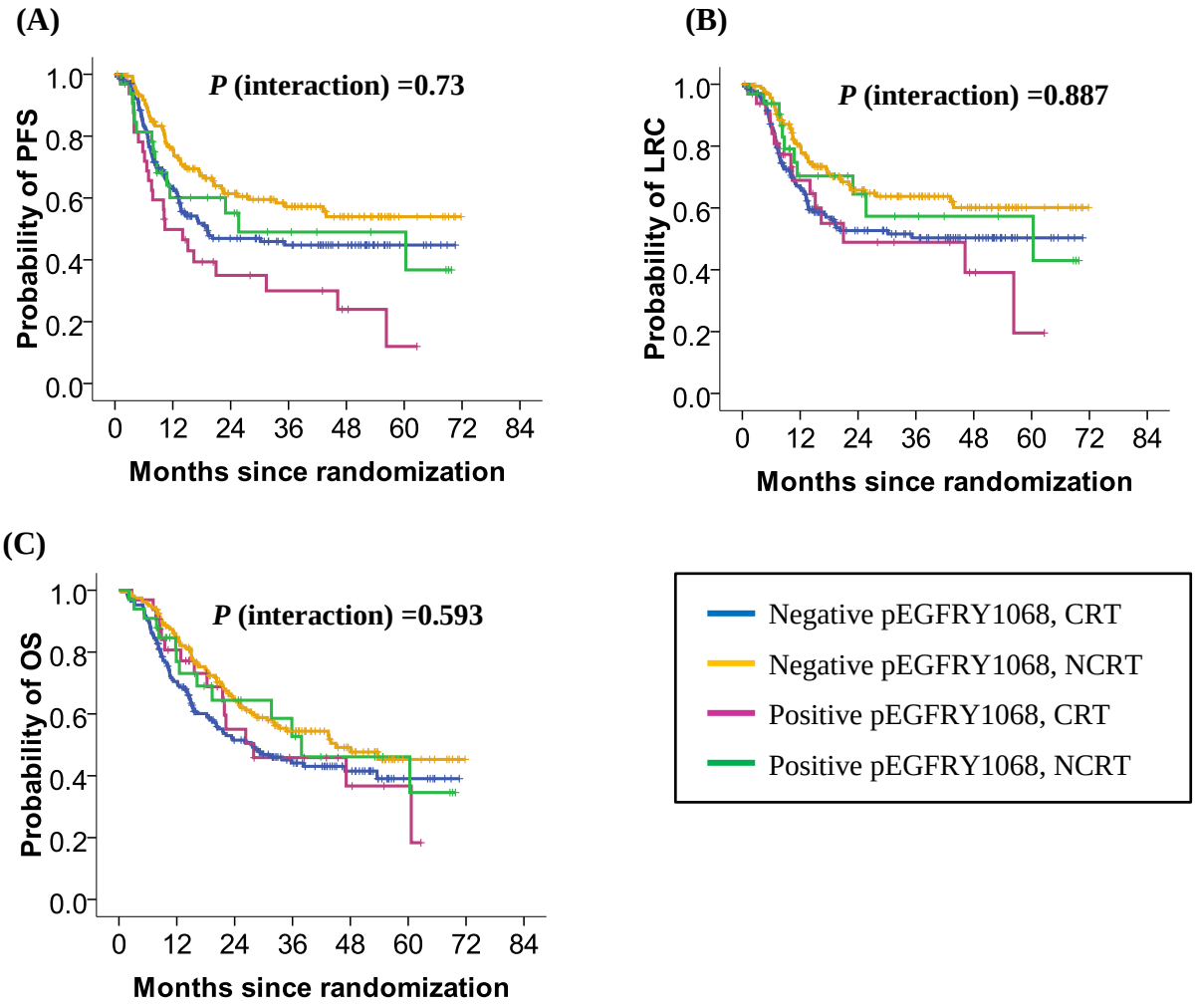



***Figure 22: Kaplan-Meier plots showing clinical outcomes according to the membrane pEGFRY1068 status and treatment.*** *PFS (A), LRC (B) and (C) OS.*

Significant longer LRC was observed in the patients with tumors lacking expression of pEGFRY1173 with NCRT versus CRT [HR (95%CI) =0.66 (0.45-0.97), *P*=0.034]. While PFS was significantly better in the patients with positive pEGFRY1173 expression with NCRT versus CRT [HR (95%CI) =0.52 (0.29-0.94), *P*=0.031]. No interaction was observed between treatment effect and pEGFRY1173 status for any of the studied endpoints (Figure 23 and Figure 25-27). It should be noted that in the current study patients with tumors expressing pEGFR dimers were small in number as a majority of the HNSCCs were negative for pEGFR expression, therefore, these results should be interpreted carefully and needs to be further validated.

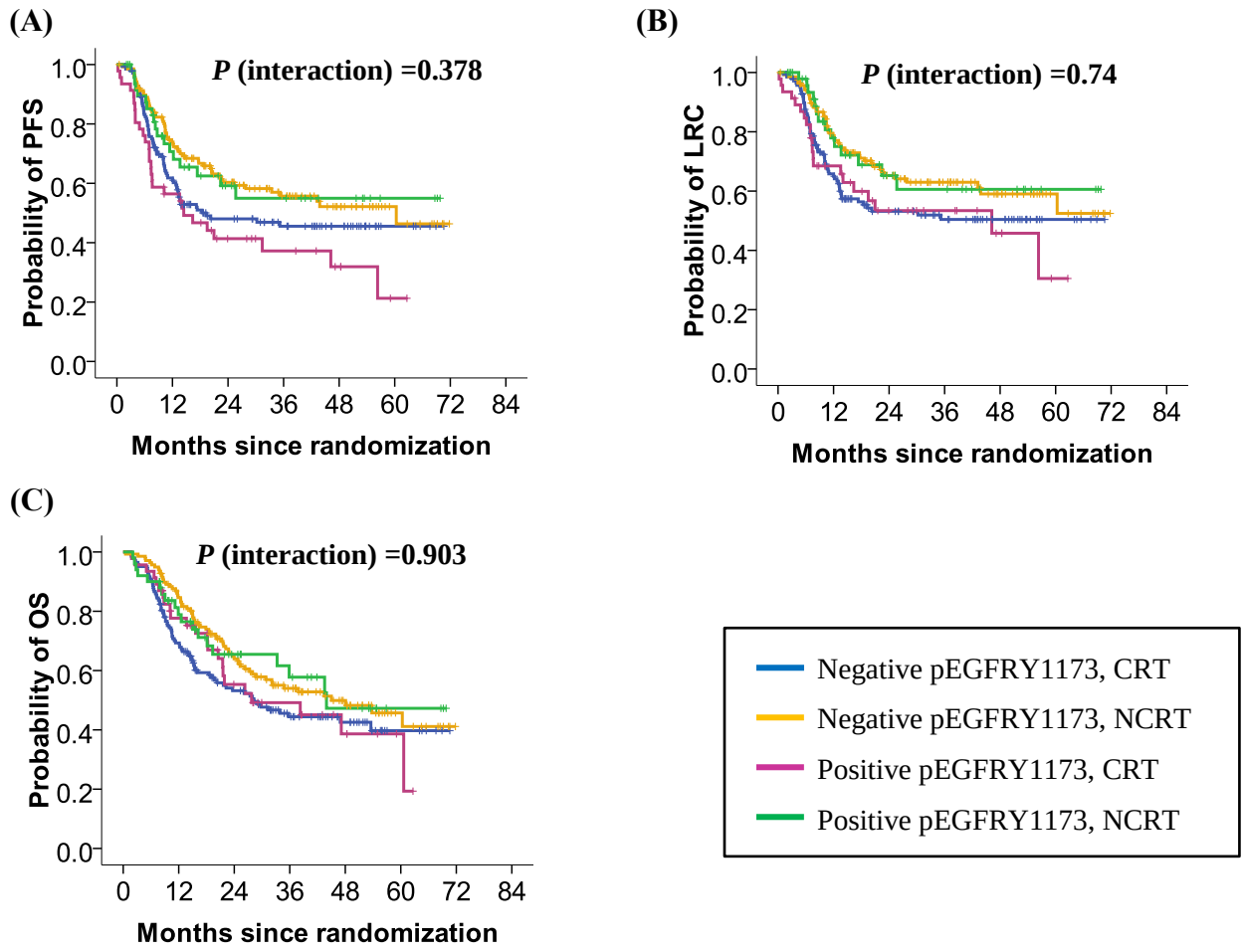



***Figure 23: Kaplan-Meier plots showing clinical outcomes according to the membrane pEGFRY1173 status and treatment.*** *PFS (A), LRC (B) and (C) OS.*

EGFR FISH negative status was significantly associated with better PFS [HR (95%CI) =0.60 (0.40-0.91), *P*=0.015] and OS [HR (95%CI) =0.68 (0.46-0.99), *P*=0.047] with NCRT versus CRT. Although, difference in LRC between the treatments was not statistically significant [HR (95%CI) =0.63 (0.33-1.22), *P*=0.167]. We did not observe similar benefits in PFS, LRC, or OS in the patients with EGFR FISH positive status with NCRT versus CRT (Figure 24).

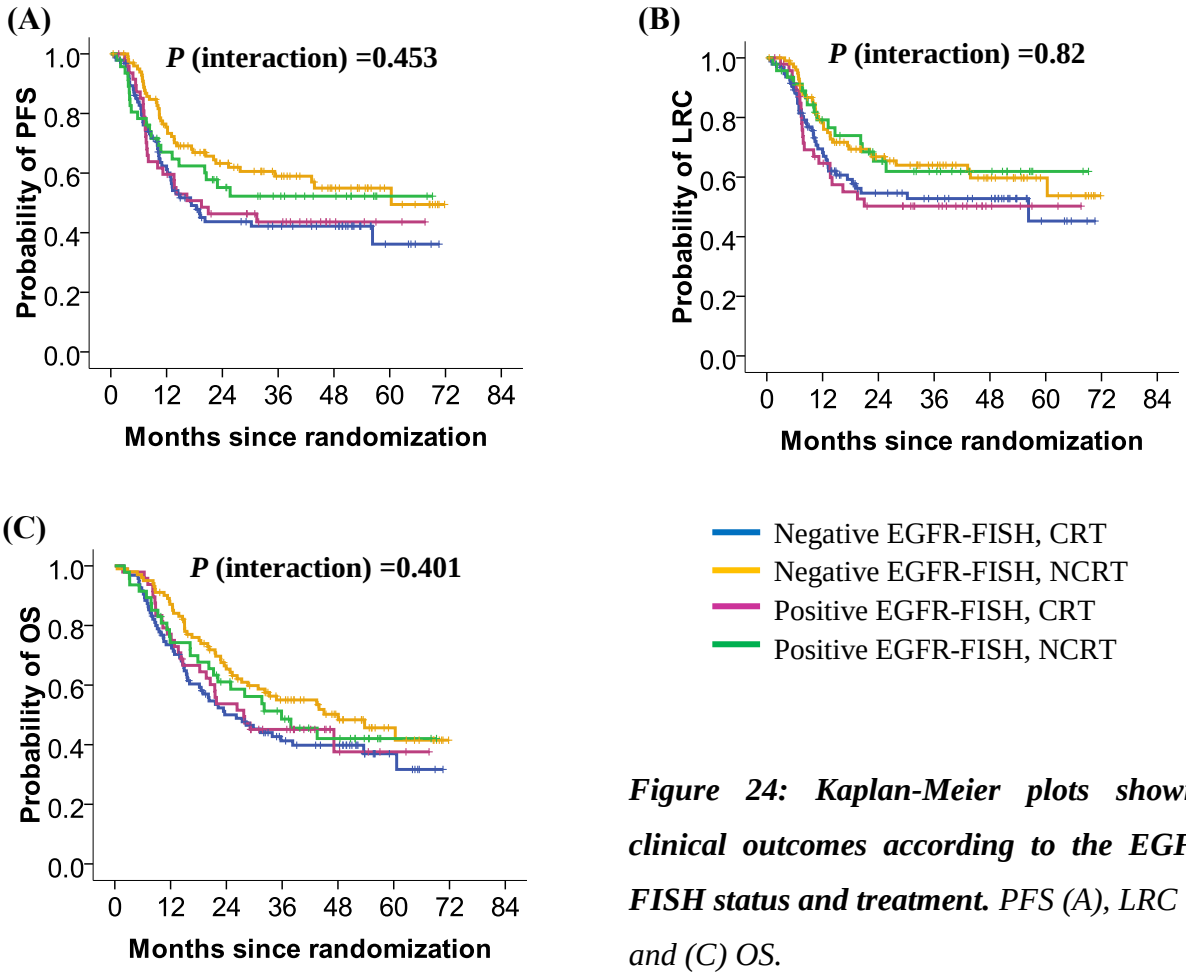



***Figure 24: Kaplan-Meier plots showing clinical outcomes according to the EGFR-FISH status and treatment.*** *PFS (A), LRC (B) and (C) OS.*

Also, the interaction between treatment effect and EGFR FISH status was found to be nonsignificant (Figure 25-27). Overall, our results suggest that the treatment effect of NCRT is independent of expression of total or pEGFR protein as well as EGFR gene copy status in these patients.

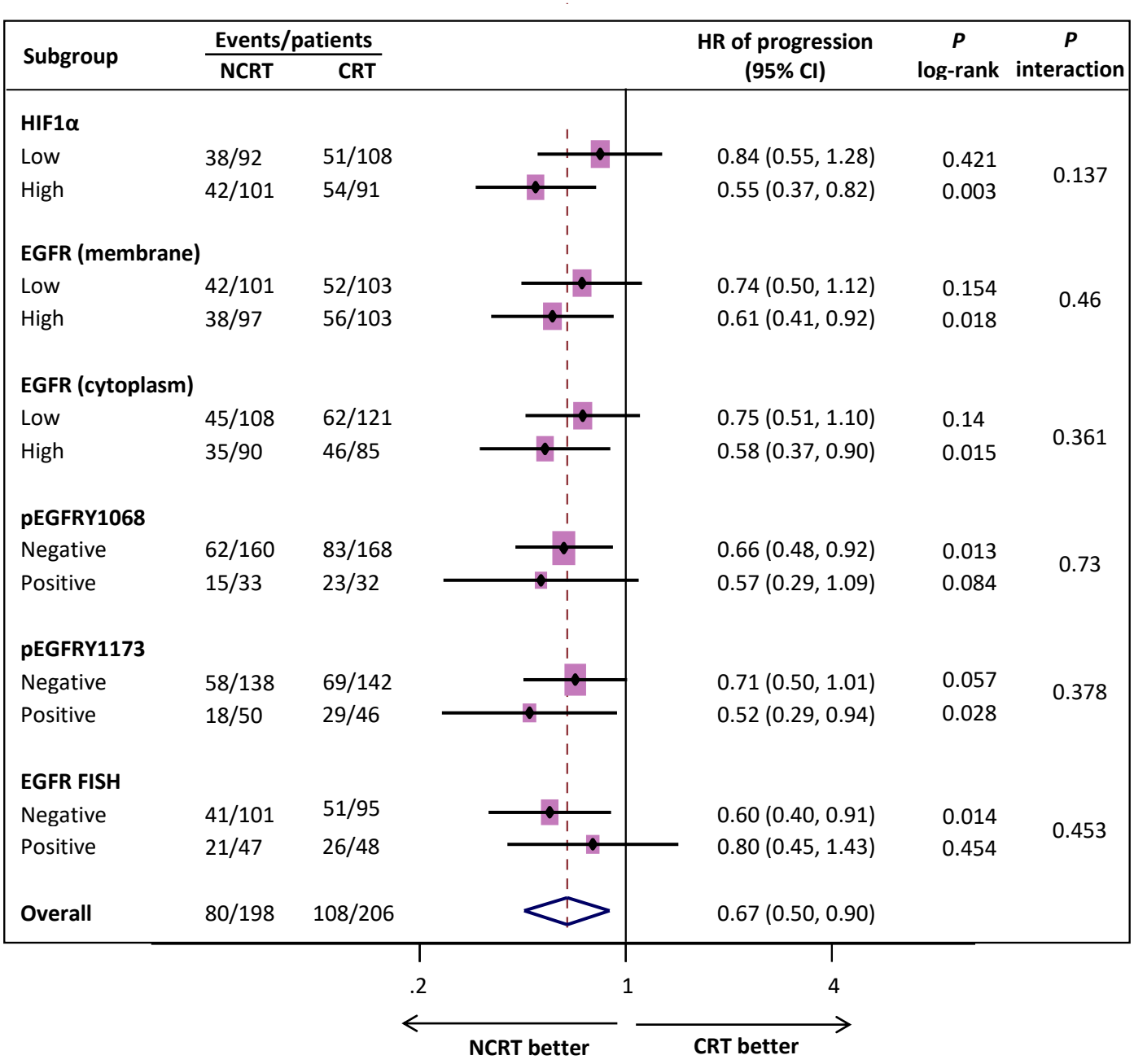



***Figure 25: Forest plot for progression free survival (PFS) by biomarker defined subgroups.*** *The interaction P value is based on a two-sided test of interaction between treatment and biomarker expression status in the Cox proportional hazards model. A hazard ratio (HR) of less than 1 indicates a benefit with the addition of nimotuzumab. The diamond represents the respective hazard ratio for the overall study population.*

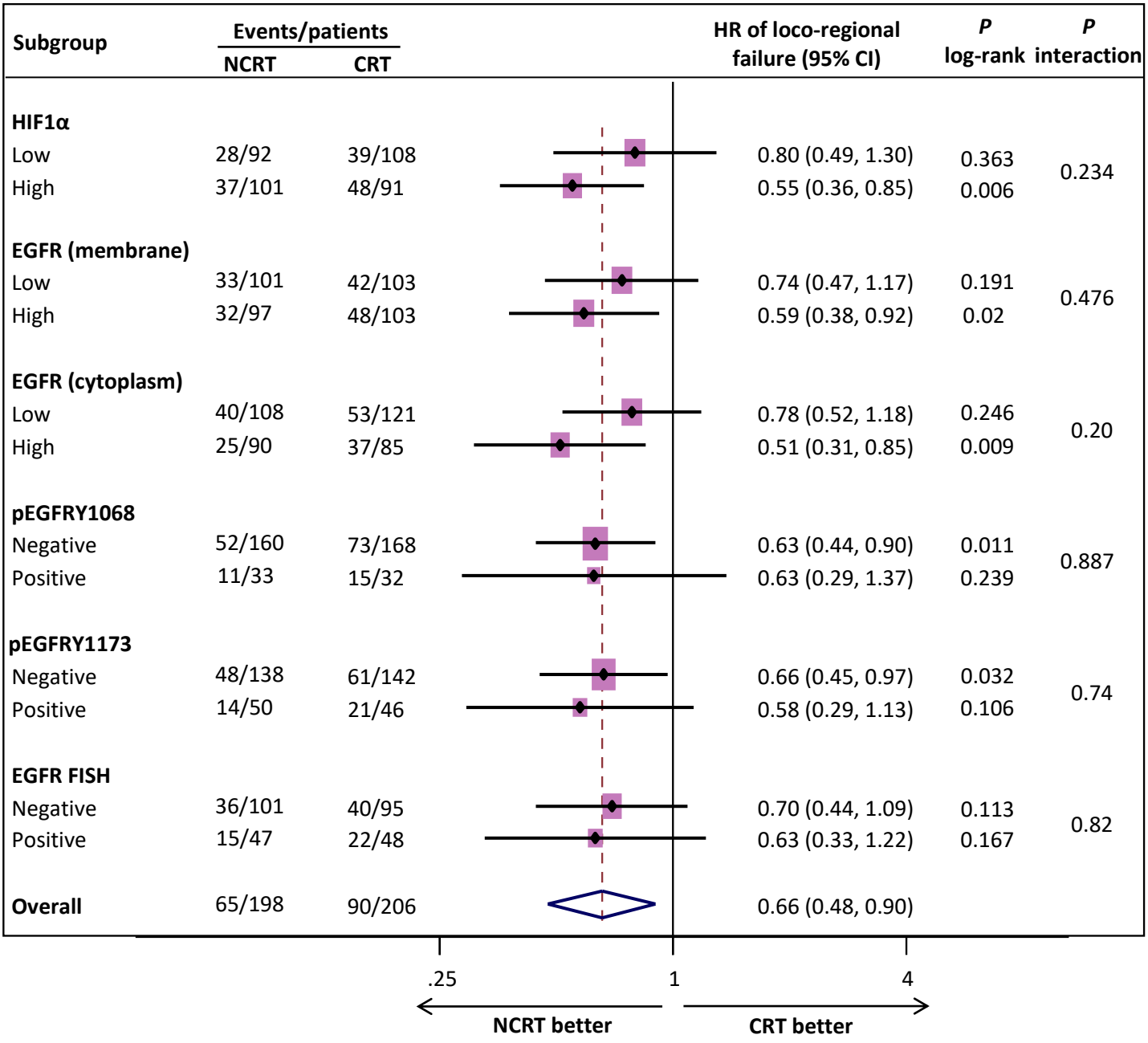



***Figure 26: Forest plot for loco-regional control (LRC) by biomarker defined subgroups.*** *The interaction P value is based on a two-sided test of interaction between treatment and biomarker expression status in the Cox proportional hazards model. A hazard ratio (HR) of less than 1 indicates a benefit with the addition of nimotuzumab. The diamond represents the respective hazard ratio for the overall study population.*

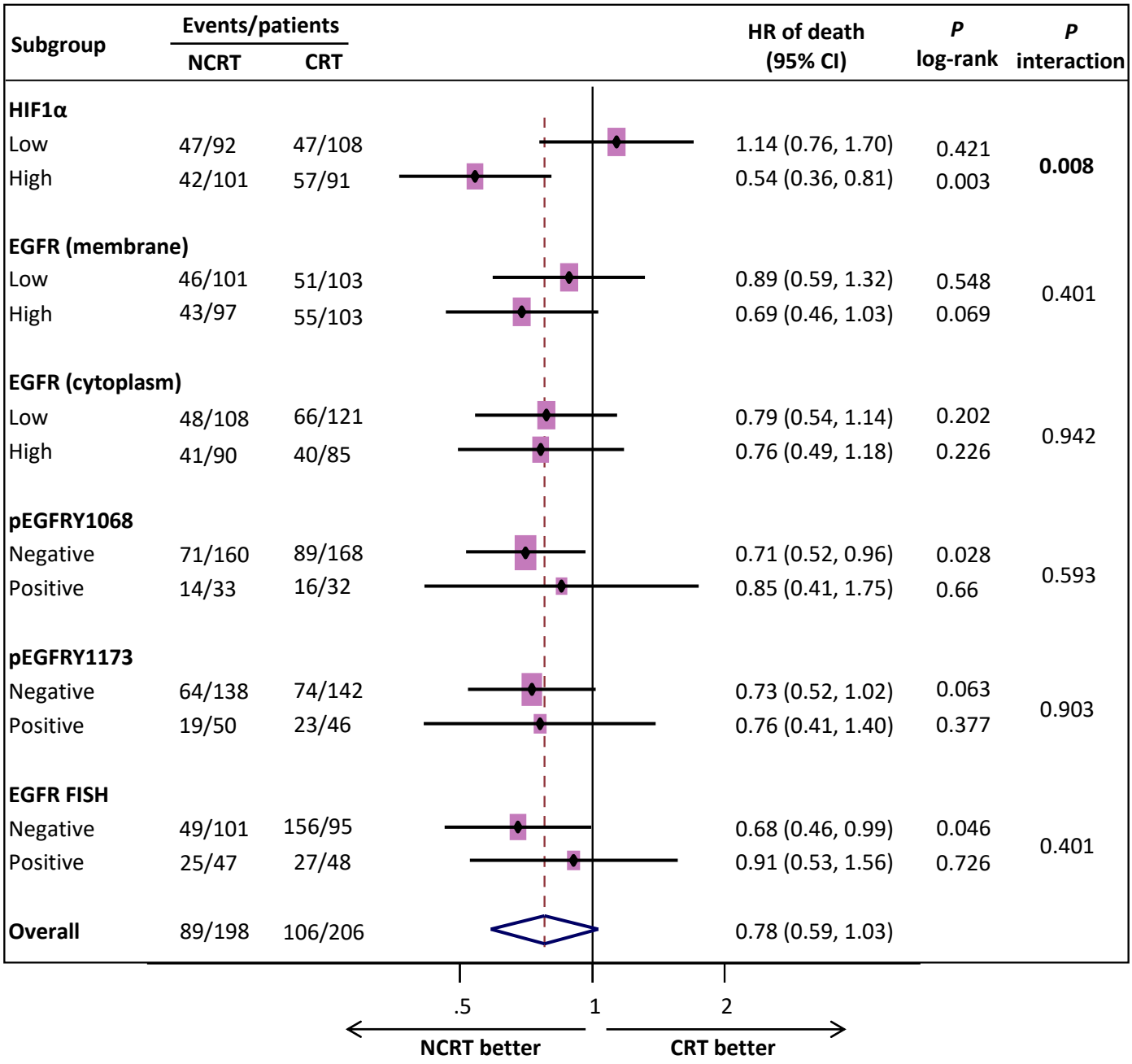



***Figure 27: Forest plot for overall survival (OS) by biomarker defined subgroups.*** *The interaction P value is based on a two-sided test of interaction between treatment and biomarker expression status in the Cox proportional hazards model. A hazard ratio (HR) of less than 1 indicates a benefit with the addition of nimotuzumab. The diamond represents the respective hazard ratio for the overall study population.*

### 4.8.4. Predictive significance of combined HIF1α and EGFR expression status

In the combined predictive analysis of HIF1α and membrane EGFR expression, we observed that the patients with tumors expressing high HIF1α and high membrane EGFR had significantly improved PFS (*P*=0.04), LRC (*P*=0.036), and OS (*P*=0.013) with NCRT versus CRT (Table 22). Patients with high HIF1α but low membrane EGFR also showed significantly longer PFS (*P*=0.036) with NCRT compared to patients treated with CRT. The difference in LRC and OS between NCRT versus CRT did not reach statistical significance in these patients. In the subgroups with either low expression of both biomarkers or low HIF1α along with high EGFR expression, no significant difference was seen in PFS, LRC or OS with NCRT versus CRT. Taken together, our combined analysis of HIF1α and membrane EGFR expression failed to show any additional predictive role over HIF1α alone.

***Table 22: Combined analysis of HIF1α and EGFR (membrane) for its predictive significance***

| Biomarker combination | Events/n (NCRT) | Events/n (CRT) | 4 YR survival (months) | HR (95% CI) | *P** | *P* (interaction) |
|---|---|---|---|---|---|---|
| **Progression free survival (PFS)** | | | | | | |
| Both low | 22/51 | 30/64 | 48.2 vs 46.8 | 0.91 (0.53-1.58) | 0.747 | 0.327 |
| HIF1α high-EGFR low | 20/48 | 21/34 | 56.8 vs 34.3 | 0.52 (0.28-0.96) | **0.036** | |
| HIF1α low-EGFR high | 16/41 | 21/44 | 50.1 vs 46.4 | 0.76 (0.40-1.47) | 0.418 | |
| Both high | 22/53 | 33/57 | 53.1 vs 36.6 | 0.57 (0.33-0.98) | **0.04** | |
| **Loco-regional control (LRC)** | | | | | | |
| Both low | 15/51 | 23/64 | 63.9 vs 58.3 | 0.82 (0.43-1.57) | 0.546 | 0.418 |
| HIF1α high-EGFR low | 18/48 | 18/34 | 60.1 vs 42.0 | 0.57 (0.29-1.09) | 0.088 | |
| HIF1α low-EGFR high | 13/41 | 16/44 | 55.5 vs 53.6 | 0.78 (0.38-1.63) | 0.51 | |
| Both high | 19/53 | 30/57 | 61.0 vs 40.6 | 0.54 (0.30-0.96) | **0.036** | |
| **Overall survival (OS)** | | | | | | |
| Both low | 25/51 | 30/64 | 42.6 vs 47.7 | 1.07 (0.63-1.82) | 0.797 | 0.127 |
| HIF1α high-EGFR low | 21/48 | 21/34 | 50.4 vs 30.9 | 0.60 (0.33-1.10) | 0.097 | |
| HIF1α low-EGFR high | 22/41 | 17/44 | 40.0 vs 54.6 | 1.23 (0.65-2.32) | 0.518 | |
| Both high | 21/53 | 36/57 | 53.5 vs 32.6 | 0.51 (0.29-0.87) | **0.013** | |

*Univariate Cox regression analysis. Respective median HScore values were used as the cutpoint for categorization [HIF1α=90; EGFR=100].

## 4.9. Summary and Discussion

We showed that high HIF1α is an independent negative prognostic factor for PFS, LRC, and OS in HNSCC patients. HIF1α status also emerged as a potential predictive biomarker showing statistically significant qualitative interaction with treatment effect for OS which was further validated by the bootstrap resampling method. We did not find prognostic and/or predictive role of EGFR based biomarkers. This is the first study presenting both prognostic and predictive impact of nuclear HIF1α expression in HPV negative LA-HNSCC patients in a randomized setting.

High expression of EGFR was associated with improved outcomes with NCRT versus CRT; however, the treatment interaction test was nonsignificant. Lack of correlation between EGFR based biomarkers and sensitivity of EGFR inhibitors can be due to complex biology of the EGFR signaling pathways and different intrinsic and extrinsic or acquired resistance mechanisms including overexpression of ligands, activation of alternative pathways, and/or alterations in downstream molecules like PI3K, AKT, mTOR which can alter the EGFR downstream signaling pathways. These resistance mechanisms of anti-EGFR therapy are well established in CRC and NSCLC but are poorly understood in HNSCCs (82, 109).

In literature, several pre-clinical studies on different cancer cell lines have shown that the response of tumor cells to EGFR inhibitors is to some extent dependent on the downregulation of HIF1α (14, 15, 79-81, 110). Additionally, i*n vivo* studies have also shown that the downregulation of HIF1α upon anti-EGFR treatment decreases levels of downstream targets of HIF1α including vascular endothelial growth factor (VEGF) which is a well-known strong pre-angiogenic factor. Downregulation of VEGF in turn leads to normalization of the vasculature and

improves blood flow which leads to enhanced chemo-radiation efficacy (15, 81). Nevertheless, the role of HIF1α or VEGF in predicting anti-EGFR based treatment response in HNSCC patients has not been studied. A study by Ou et al have reported an independent prognostic role of combined expression of low CD34 and high CA9 (carbonic anhydrase 9) in predicting poor LCR retrospectively; however, they did not observe any predictive significance of these hypoxia biomarkers in HPV negative LA-HNSCC patients (111). Also, this study was carried out in a small number of HNSCC patients with an unbalanced distribution between the two treatment groups. *In vitro* studies have also shown increased sensitivity of HNSCC cells to cetuximab under hypoxia which was efficiently reversed upon HIF-1α knock-down in HNSCC cells (16, 17). The underlying mechanism by which hypoxia or HIF1α mediates sensitization towards anti-EGFR mAbs is not yet clearly understood. In our combined analysis of EGFR and HIF1α, we showed that the improved response to NCRT treatment was independent of EGFR expression status in HNSCC patients.

It is known that hypoxia is a dynamic feature of the tumor microenvironment and assessing biomarker expression in biopsy specimens might not represent the whole tumor. However, integrating the functional imaging and serum-based biomarker can offer complementary information that can aid in the development of robust predictive biomarkers. In literature, very few reports have studied correlations between tissues or serum-based biomarkers and information obtained from functional imaging. One such prospective study by Nicolay et al have shown the association between HIF1α and CA9 expression studied by IHC in pre-treatment biopsies and hypoxia dynamics assessed by 18F-FMISO PET/CT imaging during chemoradiation in LA-HNSCC patients (112).

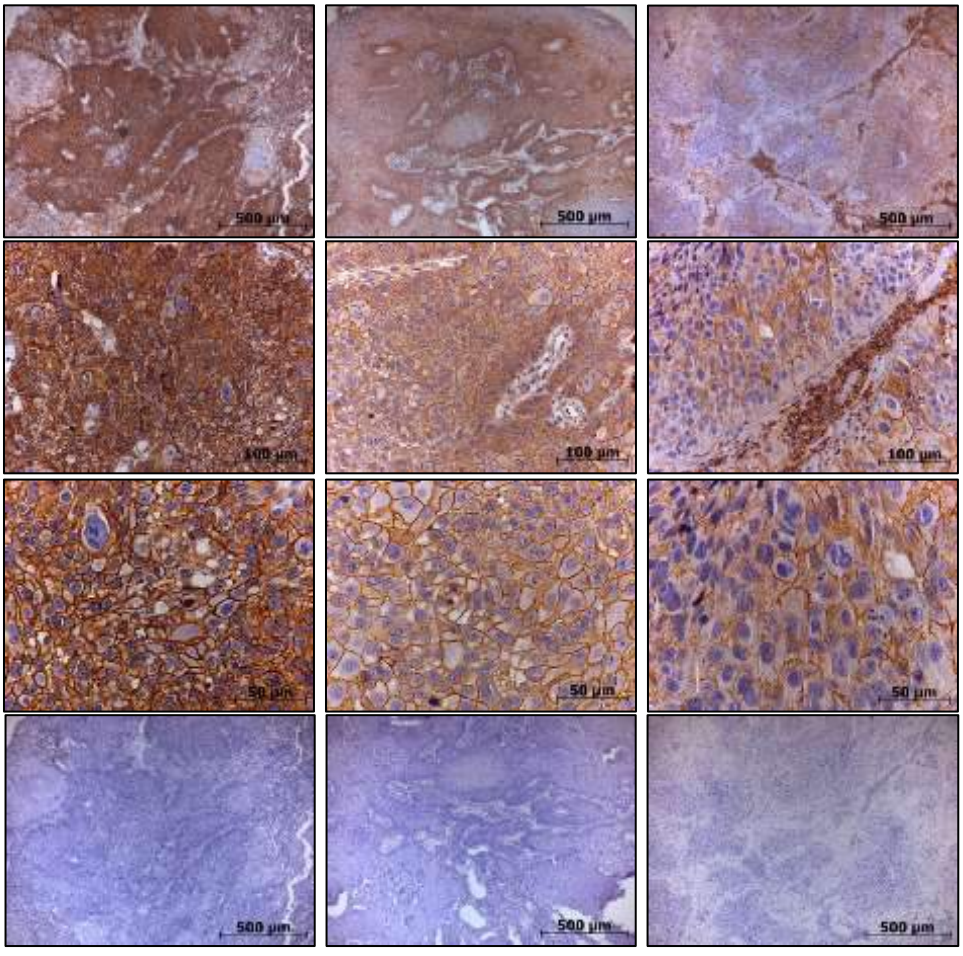
# 5.

# Results-II

# Prognostic and predictive significance of different putative cancer stem cell markers in HPV negative LA-HNSCCs

## 5.1. Expression of different biomarkers

**5.1.1. Expression of CD44 and CD44v6:** Out of 404 HPV negative patients, CD44 expression was analyzed in 383 (94.8%) patients and CD44v6 expression was analyzed in 397 (98.3%) patients. The complete membrane expression of CD44 and CD44v6 was absent in about 25.8%, and 7.6% of the HNSCCs respectively. Representative IHC staining images of CD44 and CD44v6 expression are provided in Figure 28 A-B. Frequency distribution was comparable between the two treatment arms (Figure 29 A-B).




**HScore=210** **HScore=180** **HScore=90**

***Figure 28A: Representative IHC staining results showing membrane expression of CD44****. Bottom panel shows respective negative control.*

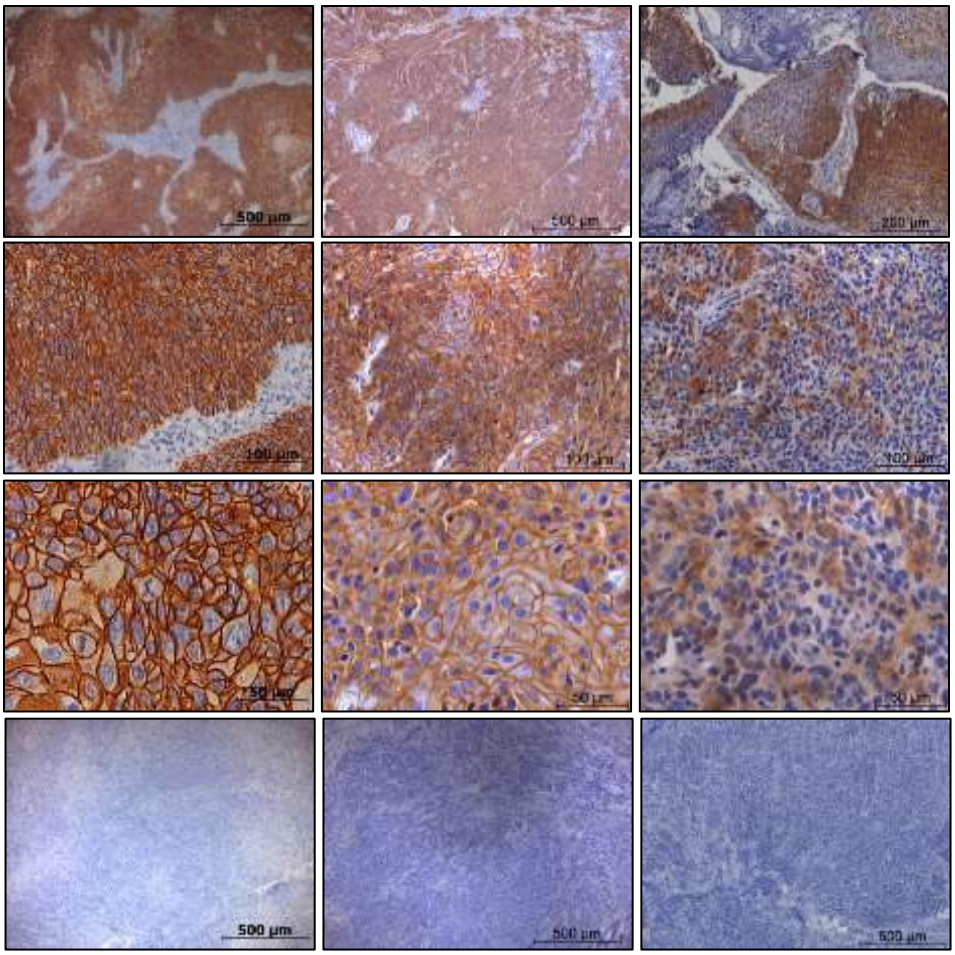
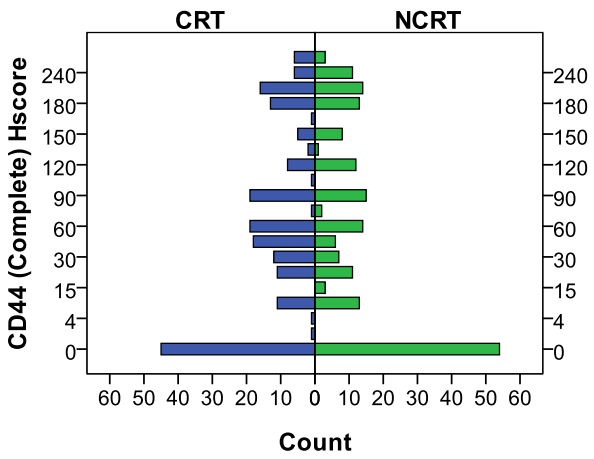


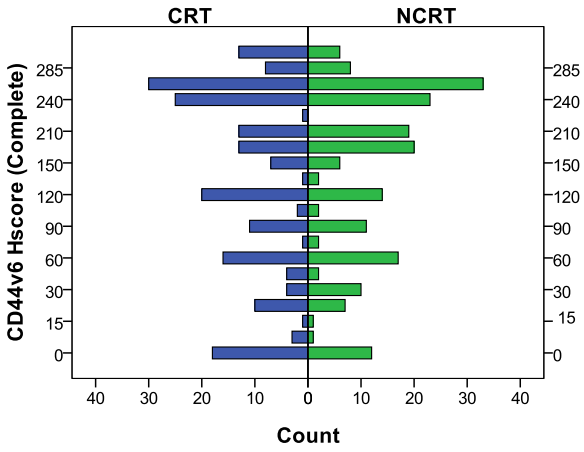

***Figure 28B: Representative IHC staining results showing membrane expression of CD44v6****. Bottom panel shows respective negative control.*

**(A)**




| CD44 | |
|---|---|
| Median=40<br>IQR=10-135<br>n=196 | Median=40<br>IQR=0-150<br>n=187 |

**(B)**




| CD44v6 | |
|---|---|
| Median=180<br>IQR=60-270<br>n=201 | Median=180<br>IQR=60-240<br>n=196 |

***Figure 29: Histograms showing frequency distribution of complete membrane expression of CD44 (A) and CD44v6 (B) across both the treatment groups.*** *IQR= inter quartile range.*

**5.1.2. Expression of CD98hc and ALDH1A1:** Expression levels of CD98hc and ALDH1A1 were analyzed in 370 (91.6%) and 354 (87.6%) HNSCC samples respectively. Representative images of IHC staining and frequency distribution of CD98hc and ALDH1A1 between the two treatment arms is given in Figure 30 and 31 respectively.

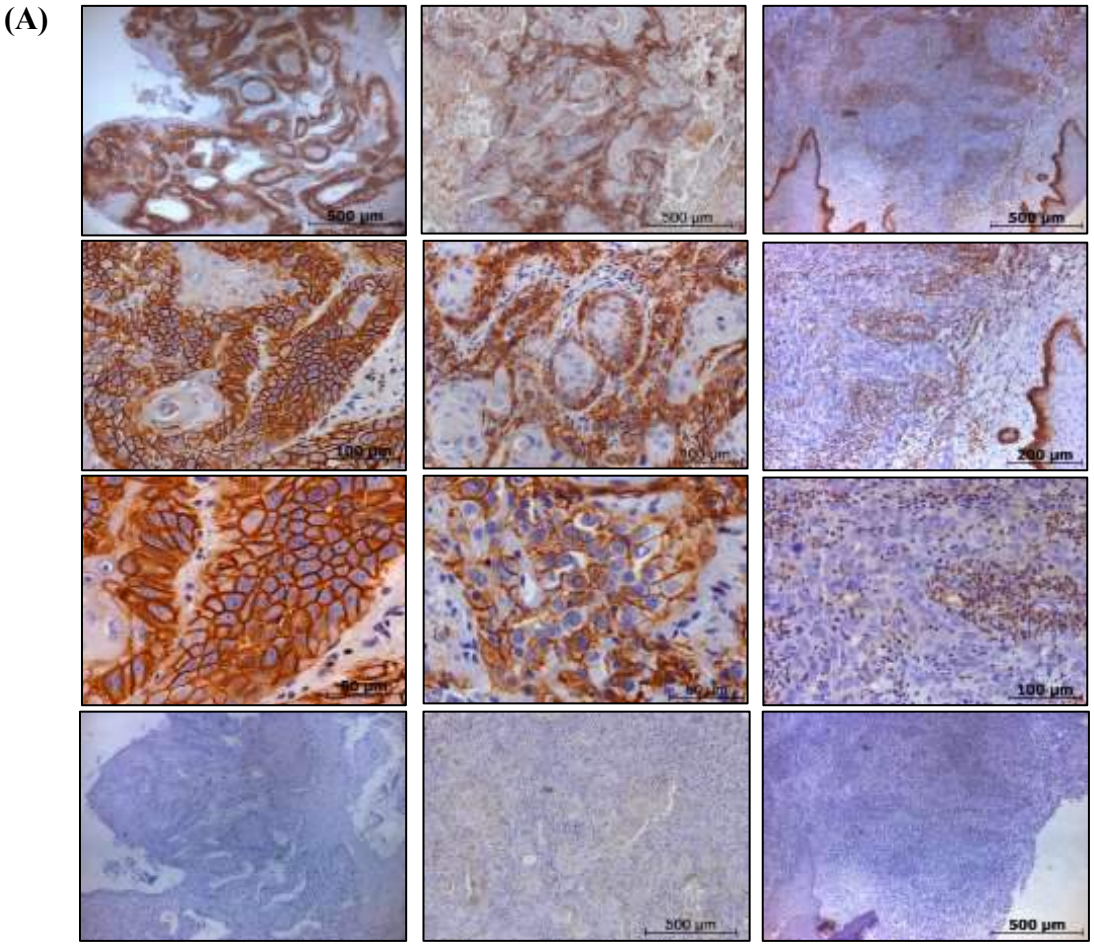
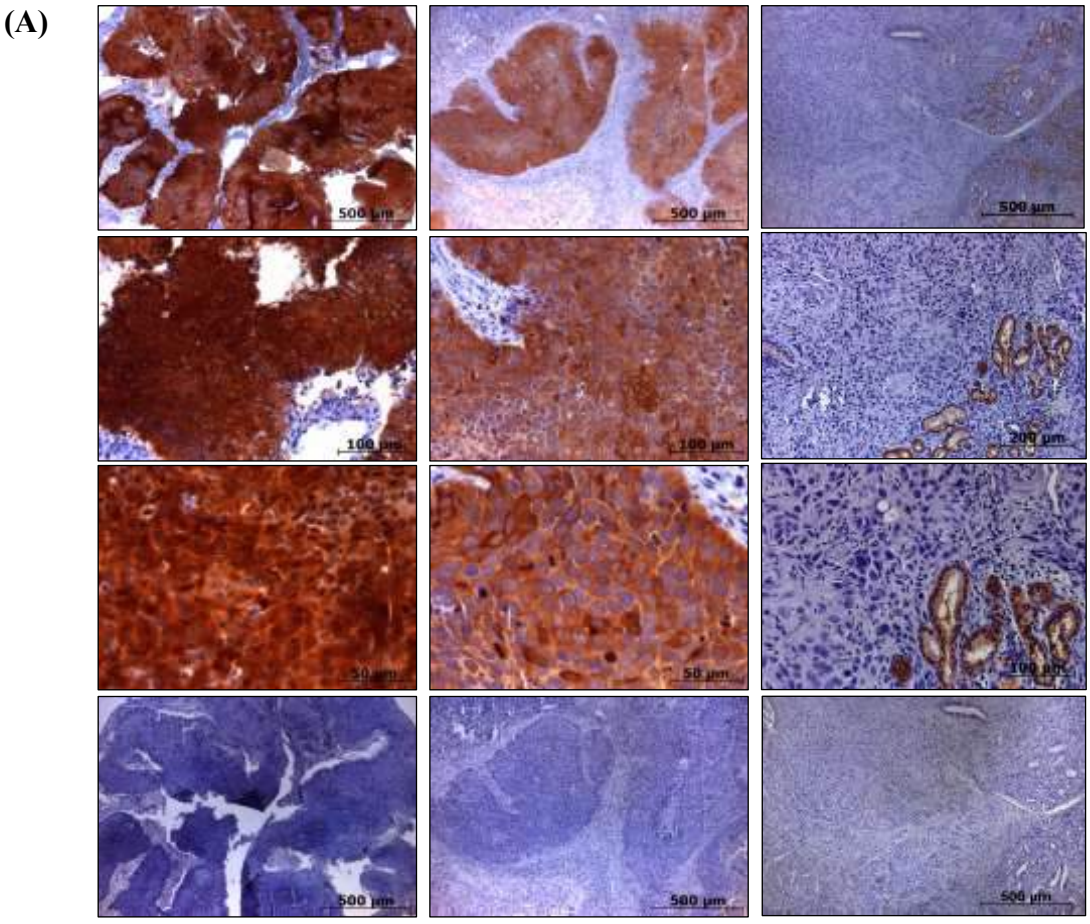



***Figure 30: Representative IHC staining (A) and frequency distribution (B) of complete membrane CD98hc expression.*** *Bottom IHC image panel shows respective negative control. IQR= inter quartile range.*



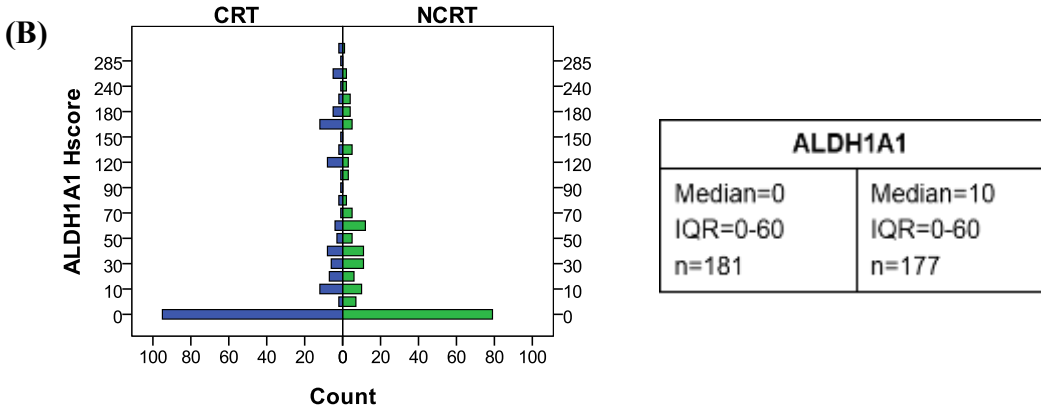
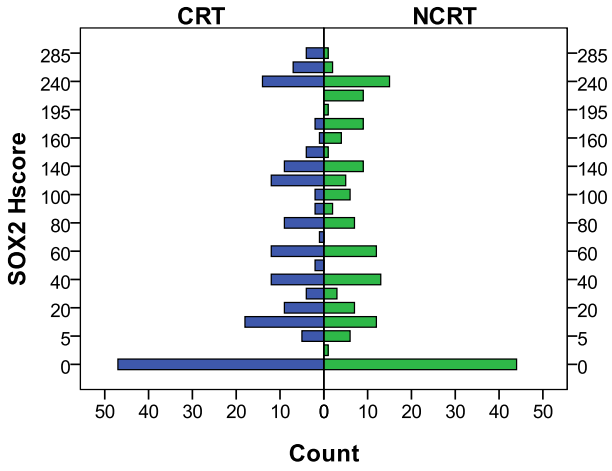




***Figure 31: Representative IHC staining (A) and frequency distribution (B) of cytoplasmic ALDH1A1 expression.*** *Bottom IHC image panel shows respective isotype control. IQR= inter quartile range.*

### 5.1.3. Expression of SOX2 and OCT4A

Nuclear SOX2 expression was analyzed in a total of 345 HNSCC tumors. About 91 (26.4%) of the tumors did not show any nuclear SOX2 staining, frequency distribution was comparable between the two treatment groups (Figure 32). We also analyzed OCT4A expression in 342 HNSCC tumors; however, none of the analyzed samples showed any nuclear OCT4A staining. Testicular seminoma tissue was used as the positive control for OCT4A staining which showed strong nuclear staining in all the batches (Figure 33).

**(A)**




| SOX2 | |
|---|---|
| Median=40<br>IQR=0-120<br>n=176 | Median=40<br>IQR=0-145<br>n=169 |

***Figure 32A: Frequency distribution of nuclear SOX2 expression.*** *IQR= inter quartile range.*

**(B)**

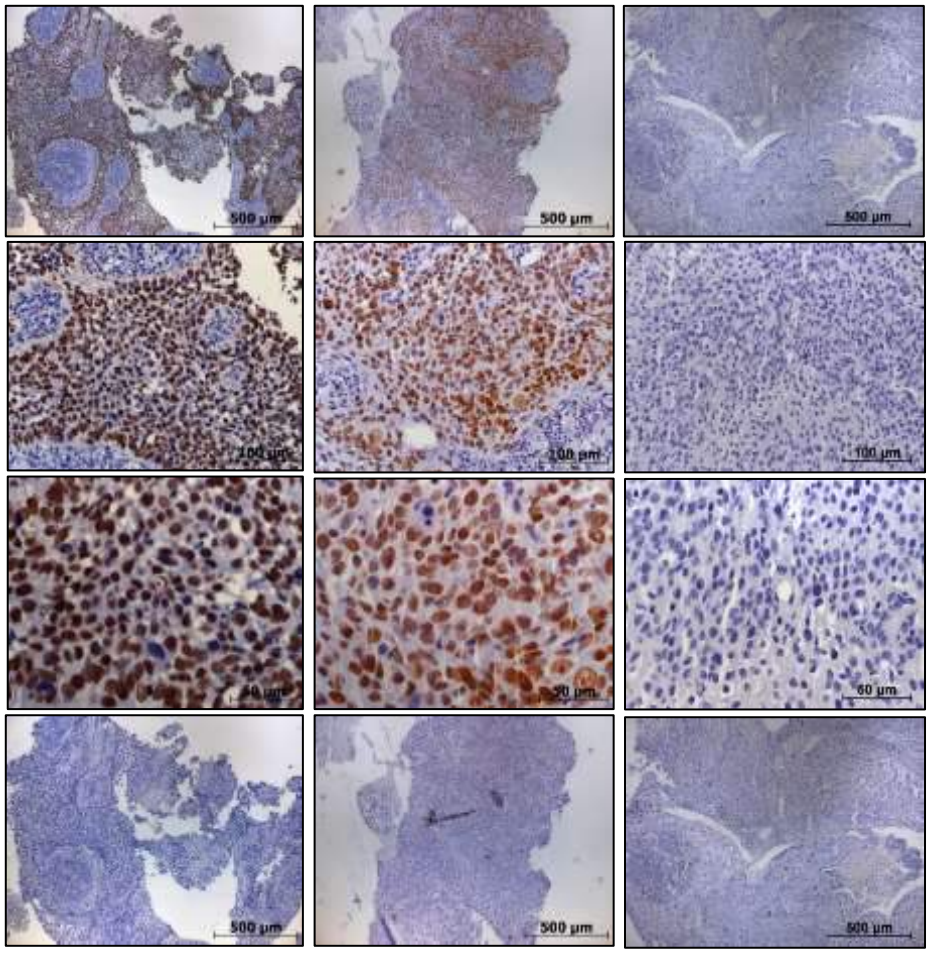



***Figure 32B: Representative IHC staining of nuclear SOX2 expression in HNSCCs.*** *Bottom IHC image panel shows respective isotype control.*

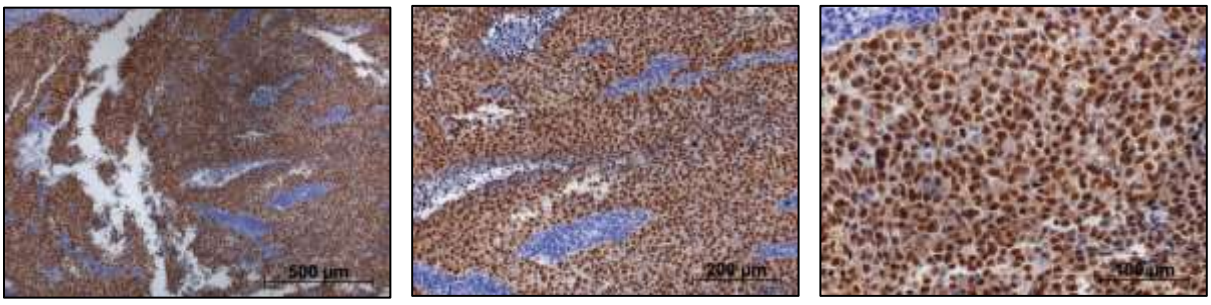



***Figure 33: Representative IHC staining of nuclear OCT4A expression in seminoma tissue***

## 5.2. Correlation among different biomarkers

A weak positive correlation was detected between CD44-CD44v6 (rho=0.45). Correlation between CD44-CD98hc (rho=0.24), CD44-EGFR (rho=0.20) and CD44-HIF1α (rho=0.21) were very weakly positive but statistically significant. CD44v6 showed very weak positive correlation with CD98hc (rho=0.24), EGFR (rho=0.15) and HIF1α (rho=0.15). Similar correlation were also observed between CD98hc-EGFR (rho=0.18) and CD98hc-HIF1α (rho=0.22). ALDH1A1 and SOX2 showed moderate positive correlation (rho=0.69). Interestingly, ALDH1A1 and SOX2 showed very weak negative correlation with CD44, EGFR, CD98hc (Table 23A, Figure 34) (113, 114).

***Table 23A: Correlation among different biomarkers (continuous HScore)***

| | | **CD44v6** | **CD98hc** | **ALDH1A1** | **SOX2** | **EGFR (Membrane)** | **HIF1α** |
|---|---|---|---|---|---|---|---|
| **CD44** | rho | **0.45**** | 0.24** | -0.12* | -0.12* | 0.20** | 0.21** |
| | *P* | 0 | 0 | 0.027 | 0.032 | 0 | 0 |
| | n | 381 | 361 | 348 | 337 | 383 | 374 |
| **CD44v6** | rho | 1 | 0.24** | -0.02 | 0 | 0.15** | 0.15** |
| | *P* | . | 0 | 0.663 | 0.993 | 0.002 | 0.003 |
| | n | 397 | 368 | 355 | 343 | 397 | 387 |
| **CD98hc** | rho | | 1 | -0.13* | -0.10 | 0.18** | 0.22** |
| | *P* | | . | 0.013 | 0.058 | 0.001 | 0 |
| | n | | 370 | 355 | 343 | 370 | 361 |
| **ALDH1A1** | rho | | | 1 | 0.**69**** | -0.12* | -0.02 |
| | *P* | | | . | 0 | 0.019 | 0.757 |
| | n | | | 358 | 336 | 358 | 350 |
| **SOX2** | rho | | | | 1 | -0.12* | -0.05 |
| | *P* | | | | . | 0.03 | 0.376 |
| | n | | | | 345 | 345 | 341 |

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed). rho= Spearman's correlation coefficient, n= number of samples

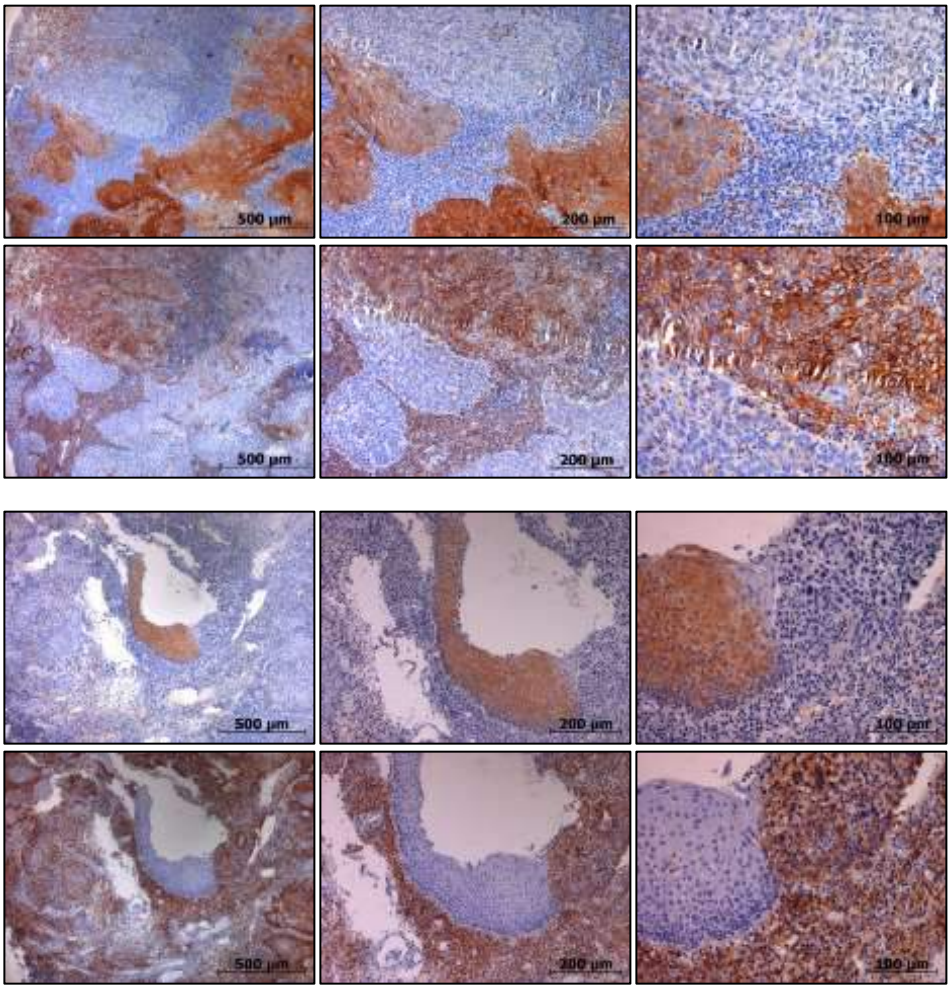



***Figure 34: HNSCC cases showing mutually exclusive expression of ALDH1A1 and CD98hc suggesting a negative correlation***

| *Table 23B: Correlation among different biomarkers (categorical)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **CD44v6** | | **CD98hc** | | **ALDH1A1** | | **SOX2** | |
| | | Low | High | Low | High | Low | High | Low | High |
| **CD44** | Low, n (%) | 70 (23.5) | 228 (76.5) | 128 (45.2) | 155 (54.8) | 215 (77.9) | 61 (22.1) | 109 (40.8) | 158 (59.2) |
| | High, n (%) | 1 (1.2) | 82 (98.8) | 24 (30.8) | 54 (69.2) | 61 (84.7) | 11 (15.3) | 43 (61.4) | 27 (38.6) |
| | *P* | <0.0001 | | 0.027 | | 0.253 | | 0.015 | |
| | R | 0.24 | | 0.12 | | -0.068 | | -0.14 | |
| **CD44v6** | Low, n (%) | | | 34 (53.1) | 30 (46.9) | 52 (86.7) | 8 (13.3) | 26 (43.3) | 34 (56.7) |
| | High, n (%) | | | 123 (40.5) | 181 (59.5) | 229 (77.6) | 66 (22.4) | 129 (45.6) | 154 (54.4) |
| | *P* | | | 0.071 | | 0.162 | | 0.324 | |
| | R | | | 0.097 | | 0.083 | | 0.054 | |
| **CD98hc** | Low, n (%) | | | | | 115 (76.7) | 35 (23.3) | 55 (37.2) | 93 (62.8) |
| | High, n (%) | | | | | 166 (81) | 39 (19) | 100 (51.3) | 95 (48.7) |
| | *P* | | | | | 0.356 | | 0.038 | |
| | R | | | | | -0.052 | | -0.11 | |
| **ALDH1A1** | Low, n (%) | | | | | | | 146 (55.3) | 118 (44.7) |
| | High, n (%) | | | | | | | 4 (5.6) | 68 (94.4) |
| | *P* | | | | | | | <0.0001 | |
| | R | | | | | | | 0.46 | |

R=Pearson's correlation coefficient; *P* values <0.05 were considered statistically significant. For categorizing biomarkers HScore cutpoint used were 150 (CD44), 40 (CD44v), 40 (CD98hc), 70 (ALDH1A1) and 40 (SOX2).

### 5.3. Association between biomarker status and patients' baseline parameters

Compared to females, males showed significantly higher expression of ALDH1A1 (*P*=0.001) and SOX2 (*P*=0.002). SOX2 was also associated with tobacco habits (*P*=0.008) and oropharynx tumor site (*P*=0.041). We did not observe other statistically significant associations between patients' clinicopathological parameters and CSC markers (Table 24).

***Table 24: Association between biomarker status and patient's baseline parameters***

| | | **Age (Years)** | | **Gender** | | **Tobacco-alcohol habit** | | **Site of tumor** | | **Clinical stage** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Below 60** | **60 or Above** | **Male** | **Female** | **No habit** | **With habit** | **Oropharynx** | **Others** | **III** | **IV** |
| **CD44** | Low, n (%) | 211 (70.3) | 89 (29.7) | 263 (87.7) | 37 (12.3) | 21 (7.1) | 273 (92.9) | 142 (47.7) | 156 (52.3) | 69 (23) | 231 (77) |
| | High, n (%) | 56 (67.5) | 27 (32.5) | 68 (81.9) | 15 (18.1) | 8 (10.3) | 70 (89.7) | 42 (50.6) | 41 (49.4) | 24 (28.9) | 59 (71.1) |
| | *P** | 0.615 | | 0.177 | | 0.362 | | 0.634 | | 0.266 | |
| **CD44v6** | Low, n (%) | 49 (67.1) | 24 (32.9) | 60 (82.2) | 13 (17.8) | 8 (11) | 65 (89) | 35 (48.6) | 37 (51.4) | 18 (24.7) | 55 (75.3) |
| | High, n (%) | 222 (68.5) | 102 (31.5) | 285 (88) | 39 (12) | 22 (7) | 291 (93) | 158 (48.9) | 165 (51.1) | 78 (24.1) | 246 (75.9) |
| | *P** | 0.817 | | 0.187 | | 0.259 | | 0.963 | | 0.916 | |
| **CD98hc** | Low, n (%) | 105 (66.9) | 52 (33.1) | 139 (88.5) | 18 (11.5) | 8 (5.2) | 146 (94.8) | 73 (46.5) | 84 (53.5) | 34 (21.7) | 123 (78.3) |
| | High, n (%) | 152 (71.4) | 61 (28.6) | 182 (85.4) | 31 (14.6) | 21 (10.2) | 184 (89.8) | 108 (51.2) | 103 (48.8) | 56 (26.3) | 157 (73.7) |
| | *P** | 0.355 | | 0.386 | | 0.082 | | 0.374 | | 0.304 | |
| **ALDH1A1** | Low, n (%) | 194 (68.3) | 90 (31.7) | 220(84) | 42 (16) | 26 (9.4) | 252 (90.6) | 136 (48.2) | 146 (51.8) | 70 (24.6) | 214 (75.4) |
| | High, n (%) | 51 (68.9) | 23 (31.1) | 93 (96.9) | 3 (3.1) | 2 (2.8) | 70 (97.2) | 39 (52.7) | 35 (47.3) | 16 (21.6) | 58 (78.4) |
| | *P** | 0.92 | | **0.001** | | 0.067 | | 0.493 | | 0.587 | |
| **SOX2** | Low, n (%) | 126 (69.6) | 55 (30.4) | 147 (81.2) | 34 (18.8) | 21 (11.9) | 155 (88.1) | 77 (42.5) | 104 (57.5) | 45 (24.9) | 136 (75.1) |
| | High, n (%) | 111 (67.7) | 53 (32.3) | 152 (92.7) | 12 (7.3) | 6 (3.7) | 155 (96.3) | 88 (53.7) | 76 (46.3) | 37 (22.6) | 127 (77.4) |
| | *P** | 0.728 | | **0.002** | | **0.008** | | **0.041** | | 0.704 | |

(*) Pearson's $\chi 2$ tests. Clinical stage is according to AJCC-UICC system (8th edition). For categorizing biomarkers HScore cutpoint used were 150 (CD44), 40 (CD44v), 40 (CD98hc), 70 (ALDH1A1) and 40 (SOX2).

## 5.4. Prognostic significance of different biomarkers

### 5.4.1. Prognostic significance of CD44 and CD44v6

Univariate Cox analysis revealed that the complete membrane expression of CD44 (median HScore=40) or CD44v6 (median HScore=180) was not associated with PFS, LRC, or OS in the CRT group. Additionally, we carried out univariate Cox analysis at other possible cutpoints of CD44 and CD44v6 expression HScore (Table 25-26).

***Table 25: Cutpoint analysis to assess the prognostic role of complete membrane CD44 HScore***

| CRT (n=196) | | | PFS | | LRC | | OS | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low** | **High** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** |
| 0 &>0 | 45 | 151 | 0.89 (0.57-1.41) | 0.62 | 0.98 (0.60-1.59) | 0.923 | 1.08 (0.69-1.67) | 0.74 |
| ≤15 &>15 | 58 | 138 | 0.88 (0.58-1.34) | 0.55 | 0.96 (0.61-1.51) | 0.863 | 1.09 (0.73-1.65) | 0.666 |
| ≤20 &>20 | 69 | 127 | 1.07 (0.72-1.58) | 0.757 | 1.02 (0.66-1.57) | 0.945 | 1.24 (0.84-1.84) | 0.281 |
| **≤40 &>40** | **99** | **97** | **1.05 (0.72-1.54)** | **0.791** | **1.11 (0.73-1.69)** | **0.621** | **1.15 (0.78-1.69)** | **0.496** |
| ≤60 &>60 | 118 | 78 | 1.01 (0.68-1.49) | 0.979 | 1.11 (0.72-1.71) | 0.65 | 1.16 (0.77-1.74) | 0.479 |
| ≤90 &>90 | 138 | 58 | 1.15 (0.75-1.76) | 0.534 | 1.13 (0.71-1.81) | 0.601 | 1.22 (0.78-1.90) | 0.391 |
| ≤120 &>120 | 147 | 49 | 1.40 (0.87-2.24) | 0.163 | 1.28 (0.77-2.13) | 0.338 | 1.46 (0.89-2.40) | 0.139 |
| ≤150 &>150 | 154 | 42 | 1.27 (0.78-2.06) | 0.343 | 1.19 (0.70-2.02) | 0.525 | 1.43 (0.84-2.44) | 0.187 |
| ≤180 &>180 | 168 | 28 | 0.89 (0.52-1.52) | 0.675 | 0.81 (0.46-1.44) | 0.47 | 1.01 (0.56-1.81) | 0.971 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval

***Table 26: Cutpoint analysis to assess the prognostic role of complete membrane CD44v6 HScore***

| CRT (n=201) | | | PFS | | LRC | | OS | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low** | **High** | **HR (95% CI)** | **P*** | **HR (95% CI)** | **P*** | **HR (95% CI)** | **P*** |
| ≤15 &>15 | 22 | 179 | 0.88 (0.47-1.65) | 0.70 | 0.77 (0.37-1.59) | 0.474 | 1.14 (0.64-2.05) | 0.652 |
| ≤30 &>30 | 36 | 165 | 1.09 (0.68-1.76) | 0.722 | 1.06 (0.63-1.81) | 0.82 | 1.24 (0.78-1.98) | 0.355 |
| ≤40 &>40 | 40 | 161 | 1.14 (0.72-1.79) | 0.583 | 1.15 (0.70-1.90) | 0.574 | 1.28 (0.82-1.99) | 0.278 |
| ≤60 &>60 | 56 | 145 | 0.92 (0.60-1.41) | 0.693 | 0.92 (0.58-1.47) | 0.732 | 1.08 (0.71-1.63) | 0.73 |
| ≤90 &>90 | 68 | 133 | 0.82 (0.55-1.24) | 0.353 | 0.82 (0.52-1.28) | 0.384 | 0.86 (0.57-1.29) | 0.463 |
| ≤120 &>120 | 90 | 111 | 1.13 (0.77-1.66) | 0.538 | 1.02 (0.67-1.56) | 0.919 | 1.13 (0.77-1.66) | 0.538 |
| ≤150 &>150 | 98 | 103 | 1.04 (0.71-1.52) | 0.84 | 1.10 (0.73-1.68) | 0.646 | 1.21 (0.83-1.79) | 0.323 |
| **≤180 &>180** | **90** | **111** | **1.16 (0.79-1.71)** | **0.447** | **1.19 (0.77-1.81)** | **0.435** | **1.28 (0.87-1.90)** | **0.215** |
| ≤210 &>210 | 77 | 124 | 1.05 (0.71-1.57) | 0.795 | 1.06 (0.69-1.64) | 0.787 | 1.22 (0.82-1.84) | 0.33 |
| ≤240 &>240 | 51 | 150 | 0.93 (0.60-1.44) | 0.748 | 1.01 (0.62-1.65) | 0.969 | 1.01 (0.64-1.58) | 0.979 |
| ≤270 &>270 | 21 | 180 | 0.78 (0.43-1.43) | 0.43 | 0.77 (0.40-1.49) | 0.442 | 0.76 (0.40-1.42) | 0.383 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval

However, CD44 or CD44v6 did not show any significant association with any of the studied endpoints at any of the cutpoints, suggesting no prognostic significance in this study.

### 5.4.2. Prognostic significance of CD98hc and ALDH1A1

At median cutpoint CD98hc (HScore=60) or ALDH1A1 (HScore=5) did not show any significant association with clinical outcomes. However, low CD98hc expression showed a trend towards longer OS (Table 27). Unadjusted univariate Cox analysis at different cutpoints of CD98hc HScore showed that at the cutpoint of 40, low CD98hc expression was significantly associated with better OS [HR (95%CI)= 0.63 (0.41-0.96), 53.9 vs 33.4 months]; however, no significant association was seen with either PFS [HR (95%CI)= 0.75 (0.50-1.13), 49.7 vs 36.3 months] or LRC [HR (95%CI)= 0.66 (0.41-1.04), 59.6 vs 43.8 months] (Table 27 and Figure 35).

***Table 27: Cutpoint analysis to assess the prognostic role of complete membrane CD98hc HScore***

| **CRT (n=188)** | | | **PFS** | | **LRC** | | **OS** | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low** | **High** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** |
| 0 &>0 | 35 | 153 | 0.55 (0.30-1.0) | 0.05 | 0.36 (0.17-0.79) | 0.011 | 0.59 (0.32-1.08) | 0.086 |
| ≤20 &>20 | 52 | 136 | 0.69 (0.43-1.11) | 0.122 | 0.58 (0.34-1.01) | 0.054 | 0.67 (0.41-1.09) | 0.103 |
| ≤40 &>40 | 77 | 111 | 0.75 (0.50-1.13) | 0.171 | 0.66 (0.41-1.04) | 0.071 | 0.63 (0.41-0.96) | 0.032 |
| **≤60 &>60** | **99** | **89** | **0.87 (0.58-1.29)** | **0.476** | **0.77 (0.50-1.20)** | **0.25** | **0.67 (0.45-1.01)** | **0.054** |
| ≤90 &>90 | 118 | 70 | 0.96 (0.64-1.44) | 0.837 | 0.86 (0.55-1.35) | 0.51 | 0.67 (0.45-1.0) | 0.051 |
| ≤120 &>120 | 136 | 52 | 0.94 (0.61-1.46) | 0.788 | 0.86 (0.54-1.40) | 0.55 | 0.69 (0.45-1.06) | 0.088 |
| ≤180 &>180 | 158 | 30 | 0.92 (0.54-1.57) | 0.749 | 0.91 (0.50-1.65) | 0.758 | 0.67 (0.41-1.11) | 0.119 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval

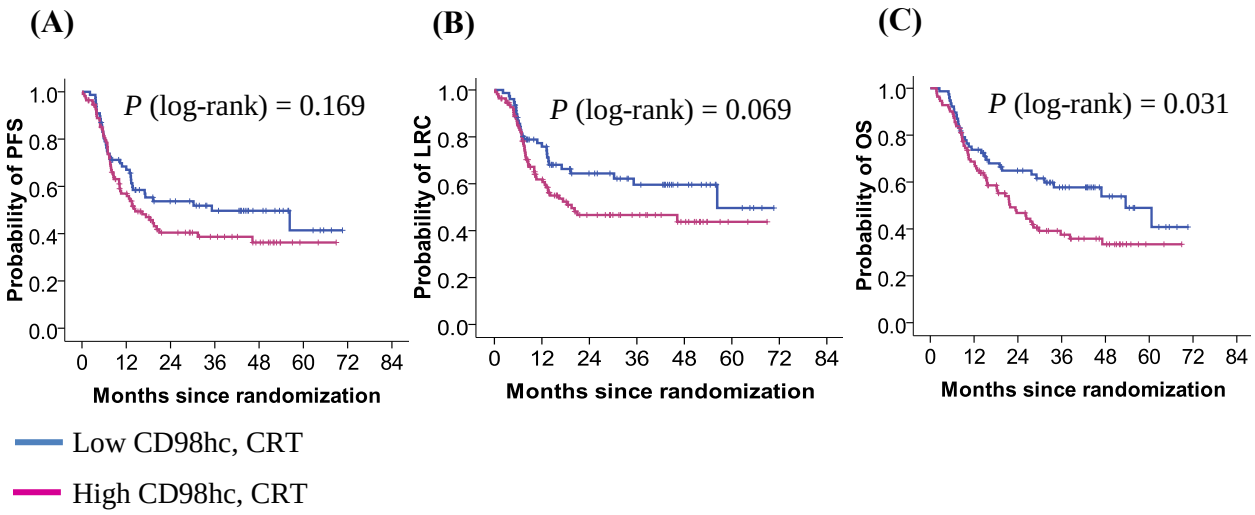



***Figure 35: Prognostic association of CD98hc expression in HNSCC.*** *Kaplan–Meier plots showing PFS (A), LRC (B), OS (C) according to CD98hc expression status (dichotomized at HScore of 40) in CRT group*

ALDH1A1 expression however did not show any association with PFS, LRC, or OS at any of the studied cutpoints (Table 28).

***Table 28: Cutpoint analysis to assess the prognostic role of cytoplasmic ALDH1A1 HScore***

| **CRT (n=181)** | | | **PFS** | | **LRC** | | **OS** | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low** | **High** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** |
| 0 & >0 | 95 | 86 | 0.84 (0.56-1.27) | 0.842 | 0.79 (0.50-1.24) | 0.306 | 0.88 (0.58-1.33) | 0.536 |
| **≤5 &>5** | **97** | **84** | **0.85 (0.56-1.27)** | **0.426** | **0.80 (0.51-1.26)** | **0.342** | **0.84 (0.55-1.26)** | **0.393** |
| ≤10 &>10 | 109 | 72 | 1.02 (0.67-1.55) | 0.927 | 1.0 (0.63-1.58) | 0.997 | 0.93 (0.61-1.42) | 0.744 |
| ≤30 &>30 | 122 | 59 | 0.96 (0.62-1.48) | 0.841 | 0.89 (0.55-1.43) | 0.631 | 1.04 (0.66-1.63) | 0.878 |
| ≤50 &>50 | 133 | 48 | 1.21 (0.74-2.0) | 0.45 | 0.94 (0.57-1.56) | 0.824 | 1.21 (0.74-2.0) | 0.45 |
| ≤70 &>70 | 138 | 43 | 1.14 (0.70-1.86) | 0.597 | 1.19 (0.70-2.05) | 0.521 | 1.50 (0.87-2.58) | 0.142 |
| ≤120 &>120 | 150 | 31 | 1.0 (0.58-1.71) | 0.494 | 1.02 (0.56-1.85) | 0.951 | 1.24 (0.67-2.27) | 0.494 |
| ≤160 &>160 | 165 | 16 | 1.20 (0.56-2.60) | 0.643 | 1.14 (0.50-2.63) | 0.756 | 1.31 (0.57-3.0) | 0.525 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval

### 5.4.3. Prognostic significance of SOX2

At median cutpoint, SOX2 (HScore=40) did not show any significant association with PFS, LRC, or OS. Additionally, SOX2 expression did not show any association with clinical outcomes at any of the studied cutpoints (Table 29).

***Table 29: Cutpoint analysis to assess the prognostic role of nuclear SOX2 HScore***

| CRT (n=176) | | | PFS | | LRC | | OS | |
|---|---|---|---|---|---|---|---|---|
| **Cutpoint** | **Low** | **High** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** | **HR (95% CI)** | ***P**** |
| 0 & >0 | 47 | 129 | 1.13 (0.71-1.80) | 0.597 | 1.04 (0.62-1.75) | 0.885 | 1.31 (0.84-2.06) | 0.231 |
| ≤10 &>10 | 70 | 106 | 1.01 (0.66-1.53) | 0.97 | 0.96 (0.60-1.54) | 0.867 | 1.19 (0.78-1.80) | 0.424 |
| ≤30 &>30 | 83 | 93 | 0.99 (0.66-1.50) | 0.977 | 0.81 (0.51-1.29) | 0.381 | 1.09 (0.72-1.64) | 0.695 |
| **≤40 &>40** | **95** | **81** | **1.08 (0.72-1.64)** | **0.703** | **0.87 (0.55-1.37)** | **0.538** | **1.12 (0.74-1.70)** | **0.599** |
| ≤60 &>60 | 109 | 67 | 1.05 (0.69-1.62) | 0.809 | 0.92 (0.58-1.47) | 0.732 | 1.01 (0.66-1.56) | 0.952 |
| ≤90 &>90 | 121 | 55 | 1.21 (0.77-1.92) | 0.415 | 1.08 (0.66-1.78) | 0.767 | 1.05 (0.66-1.65) | 0.841 |
| ≤120 &>120 | 135 | 41 | 1.25 (0.74-2.12) | 0.402 | 1.12 (0.64-1.98) | 0.694 | 1.04 (0.62-1.75) | 0.875 |
| ≤150 &>150 | 148 | 28 | 1.39 (0.74-2.61) | 0.308 | 1.16 (0.60-2.26) | 0.662 | 1.41 (0.73-2.71) | 0.311 |
| ≤180 &>180 | 151 | 25 | 1.24 (0.66-2.32) | 0.509 | 1.04 (0.53-2.02) | 0.913 | 1.44 (0.72-2.87) | 0.299 |

*Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval

### 5.4.4. Multivariable Cox analysis to find independent prognostic biomarker

After adjusting for confounding variables with a univariate Cox analysis $P< 0.20$ (Table 16), low expression of CD98hc (dichotomized at HScore=40) significantly associated with improved LRC [HR (95%CI)= 0.63 (0.39-1.0), $P$=0.049] and OS [HR (95%CI)= 0.62 (0.40-0.95), $P$=0.028] compared to high CD98hc expression in multivariate cox analysis (Table 30). Results suggest that CD98hc complete membrane expression is an independent negative prognostic factor in HPV negative LA-HNSCC patients.

***Table 30: Multivariable Cox analysis of clinical parameters and biomarkers (Model 1)***

| Variables | Univariate Cox analysis | | Multivariable Cox analysis* | |
|---|---|---|---|---|
| | **HR (95% CI)** | ***P* value** | **HR (95% CI)** | ***P* value** |
| **Progression free survival (PFS)** | | | | |
| Age (below 60 vs 60 & above) | 1.46 (0.94-2.28) | 0.092 | 1.51 (0.94-2.43) | 0.091 |
| Clinical stage (III vs IV)[#] | 0.48 (0.30-0.78) | 0.003 | 0.46 (0.27-0.77) | 0.003 |
| Site of tumor (oropharynx vs others) | 1.74 (1.19-2.56) | 0.004 | - | - |
| CD98hc (low vs high) | 0.75 (0.50-1.13) | 0.171 | - | - |
| **Loco-regional control (LRC)** | | | | |
| Age (below 60 vs 60 & above) | 1.49 (0.91-2.43) | 0.111 | 1.56 (0.91-2.68) | 0.105 |
| Clinical stage (III vs IV)[#] | 0.43 (0.25-0.75) | 0.003 | 0.39 (0.21-0.70) | 0.002 |
| Site of tumor (oropharynx vs others) | 1.58 (1.05-2.40) | 0.030 | - | - |
| CD98hc (low vs high) | 0.66 (0.41-1.04) | 0.071 | **0.63 (0.39-1.0)** | **0.049** |
| **Overall Survival (OS)** | | | | |
| Age (below 60 vs 60 & above) | 1.59 (1.0-2.53) | 0.049 | 1.55 (0.95-2.55) | 0.082 |
| Clinical stage (III vs IV)[#] | 0.64 (0.40-1.00) | 0.051 | - | - |
| Site of tumor (oropharynx vs others) | 1.62 (1.10-2.37) | 0.014 | 1.60 (1.06-2.40) | 0.025 |
| CD98hc (low vs high) | 0.63 (0.41-0.96) | 0.032 | **0.62 (0.40-0.95)** | **0.028** |

* A multivariate Cox model using backward likelihood ratio method was applied to adjust for potential confounders (clinical characteristics associated with PFS, LRC or OS at $P<0.20$ in univariate analysis). HR=hazard ratio; CI=confidence interval. (-) data not available; [#]According to AJCC-UICC system (8$^{th}$ edition).

Since previously we observed an independent prognostic impact of HIF1α expression, we created a second multivariable model with HIF1α and pEGFR dimers (Table 31). We observed that low HIF1α expression was strongly associated with improved PFS [HR (95%CI)= 0.64 (0.42-0.97), *P*= 0.033], LRC [HR (95%CI)= 0.57 (0.36-0.89), *P*=0.014] and OS [HR (95%CI)= 0.61 (0.41-0.93), *P*= 0.02]. CD98hc however did not emerge as an independent prognosticator for LRC and OS in this multivariable model. These results indicate that both HIF1α and CD98hc

are negative prognostic biomarkers; however, the prognostic impact of HIF1α expression is stronger than CD98hc.

***Table 31: Multivariable Cox analysis of clinical parameters and biomarkers (Model 2)***

| Variables | Univariate Cox analysis | | Multivariable Cox analysis* | |
|---|---|---|---|---|
| | **HR (95% CI)** | ***P* value** | **HR (95% CI)** | ***P* value** |
| **Progression free survival (PFS)** | | | | |
| Age (below 60 vs 60 & above) | 1.46 (0.94-2.28) | 0.092 | 1.59 (0.97-2.62) | 0.067 |
| Clinical stage (III vs IV) [#] | 0.48 (0.30-0.78) | 0.003 | 0.43 (0.25-0.73) | 0.002 |
| Site of tumor (oropharynx vs others) | 1.74 (1.19-2.56) | 0.004 | - | - |
| pEGFRY1068 (negative vs positive) | 0.63 (0.40-1.0) | 0.048 | - | - |
| pEGFRY1173 (negative vs positive) | 0.74 (0.48-1.14) | 0.170 | - | - |
| HIF1α (low vs high) | 0.69 (0.47-1.01) | 0.053 | **0.64 (0.42-0.97)** | **0.033** |
| CD98hc (low vs high) | 0.75 (0.50-1.13) | 0.171 | - | - |
| **Loco-regional control (LRC)** | | | | |
| Age (below 60 vs 60 & above) | 1.49 (0.91-2.43) | 0.111 | 1.59 (0.92-2.73) | 0.095 |
| Clinical stage (III vs IV) [#] | 0.43 (0.25-0.75) | 0.003 | 0.37 (0.20-0.69) | 0.002 |
| Site of tumor (oropharynx vs others) | 1.58 (1.05-2.40) | 0.030 | - | - |
| HIF1α (low vs high) | 0.58 (0.38-0.89) | 0.011 | **0.57 (0.36-0.89)** | **0.014** |
| CD98hc (low vs high) | 0.66 (0.41-1.04) | 0.071 | - | - |
| **Overall survival (OS)** | | | | |
| Age (below 60 vs 60 & above) | 1.59 (1.0-2.53) | 0.049 | 1.57 (0.96-2.58) | 0.075 |
| Clinical stage (III vs IV) [#] | 0.64 (0.40-1.00) | 0.051 | 0.59 (0.36-0.96) | 0.034 |
| Site of tumor (oropharynx vs others) | 1.62 (1.10-2.37) | 0.014 | - | - |
| HIF1α (low vs high) | 0.62 (0.42-0.91) | 0.016 | **0.61 (0.41-0.93)** | **0.020** |
| CD98hc (low vs high) | 0.63 (0.41-0.96) | 0.032 | - | - |

* A multivariate Cox model using backward likelihood ratio method was applied to adjust for potential confounders (clinical characteristics associated with PFS, LRC or OS at $P<0.20$ in univariate analysis). HR=hazard ratio; CI=confidence interval. (-) data not available; [#]According to AJCC-UICC system ($8^{th}$ edition).

## 5.5. Predictive significance of different biomarkers

### 5.5.1. Predictive significance of CD44 complete membrane expression

Hazard ratios for disease progression, loco-regional failure, and death for patients treated with NCRT relative to CRT with tumors expressing low or high CD44 dichotomized at different possible cutpoints are summarized in Table 32. Hazard ratios for all the three clinical endpoints for CD44 low expressing patients at most of the cutpoints were significantly low which suggests benefit with NCRT treatment relative to CRT. Hazard ratios for disease progression and loco-regional failure were significantly low for CD44 high expressing patients when dichotomized at the lower cutpoints. For patients with CD44 high expression defined at higher cutpoints, hazard ratios were not significantly low and for OS hazard ratios were >1.0 at higher cutpoints although they did not reach statistical significance. Interestingly, we observed statistically significant qualitative interaction between CD44 status at the cutpoints of 140 and treatment effect for OS ($P$=0.022, Table 33) and 150 ($P$=0.009, Figure 36 A-C) using interaction test.

***Table 32: Cutpoint analysis to assess the predictive role of CD44 (complete membrane) expression HScore***

| CD44 low | | | | | | CD44 high | | | | | | *P* Interaction |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **cut point** | **NCRT (n)** | **CRT (n)** | **4 YR survival (months)** | **HR (95%CI) (NCRT VS CRT)** | ***P**** | **cut point** | **NCRT (n)** | **CRT (n)** | **4 YR survival (months)** | **HR (95%CI) (NCRT VS CRT)** | ***P**** | |
| **Progression free survival (PFS)** | | | | | | | | | | | | |
| 0 | 54 | 45 | 40.1 vs 44.2 | 0.88 (0.50-1.52) | 0.634 | >0 | 133 | 151 | 59.0 vs 39.1 | 0.56 (0.39-0.80) | 0.001 | 0.157 |
| ≤10 | 67 | 58 | 48.7 vs 44.8 | 0.74 (0.45-1.23) | 0.248 | >10 | 120 | 138 | 56.3 vs 38.0 | 0.60 (0.41-0.86) | 0.006 | 0.453 |
| ≤20 | 81 | 69 | 48.7 vs 40.9 | 0.65 (0.41-1.03) | 0.064 | >20 | 106 | 127 | 57.4 vs 39.6 | 0.62 (0.42-0.93) | 0.019 | 0.863 |
| ≤30 | 88 | 81 | 48.4 vs 41.0 | 0.66 (0.43-1.01) | 0.054 | >30 | 99 | 115 | 58.1 vs 39.4 | 0.62 (0.41-0.94) | 0.023 | 0.825 |
| **≤40** | **94** | **99** | **50.6 vs 40.8** | **0.69 (0.47-1.01)** | **0.058** | **>40** | **93** | **97** | **56.2 vs 39.0** | **0.87 (0.57-1.34)** | **0.527** | **0.459** |
| ≤60 | 108 | 118 | 49.7 vs 39.5 | 0.68 (0.46-0.99) | 0.042 | >60 | 79 | 78 | 57.7 vs 41.4 | 0.60 (0.37-0.97) | 0.037 | 0.648 |
| ≤90 | 125 | 138 | 51.8 vs 38.7 | 0.61 (0.43-0.87) | 0.006 | >90 | 62 | 58 | 55.7 vs 44.3 | 0.74 (0.43-1.27) | 0.269 | 0.589 |
| ≤140 | 138 | 149 | 51.7 vs 38.4 | 0.61 (0.43-0.85) | 0.004 | >140 | 49 | 47 | 56.7 vs 45.9 | 0.82 (0.44-1.53) | 0.528 | 0.447 |
| ≤150 | 146 | 154 | 53.4 vs 38.9 | 0.59 (0.42-0.82) | 0.002 | >150 | 41 | 42 | 50.9 vs 44.7 | 0.91 (0.47-1.75) | 0.768 | 0.249 |
| ≤180 | 159 | 168 | 53.1 vs 41.6 | 0.64 (0.46-0.88) | 0.006 | >180 | 28 | 28 | 49.7 vs 35.0 | 0.70 (0.32-1.51) | 0.363 | 0.827 |
| **Loco regional control (LRC)** | | | | | | | | | | | | |
| 0 | 54 | 45 | 50.3 vs 50.7 | 0.70 (0.38-1.30) | 0.257 | >0 | 133 | 151 | 64.7 vs 47.3 | 0.59 (0.40-0.87) | 0.007 | 0.570 |
| ≤10 | 67 | 58 | 57.4 vs 51.7 | 0.63 (0.36-1.10) | 0.106 | >10 | 120 | 138 | 62.8 vs 46.0 | 0.61 (0.41-0.92) | 0.019 | 0.904 |
| ≤20 | 81 | 69 | 56.8 vs 50.4 | 0.61 (0.36-1.01) | 0.056 | >20 | 106 | 127 | 64.4 vs 46.5 | 0.62 (0.40-0.95) | 0.030 | 0.999 |
| ≤30 | 88 | 81 | 55.9 vs 48.9 | 0.61 (0.38-0.97) | 0.038 | >30 | 99 | 115 | 65.6 vs 46.9 | 0.62 (0.39-0.98) | 0.039 | 0.978 |
| **≤40** | **94** | **99** | **57.8 vs 47.8** | **0.58 (0.37-0.91)** | **0.018** | **>40** | **93** | **97** | **64.2 vs 47.6** | **0.66 (0.41-1.07)** | **0.090** | **0.733** |
| ≤60 | 108 | 118 | 57.2 vs 45.9 | 0.61 (0.40-0.92) | 0.019 | >60 | 79 | 78 | 65.3 vs 51.8 | 0.64 (0.38-1.10) | 0.106 | 0.926 |
| ≤90 | 125 | 138 | 60.3 vs 47.1 | 0.56 (0.37-0.83) | 0.004 | >90 | 62 | 58 | 59.7 vs 50.4 | 0.80 (0.44-1.43) | 0.446 | 0.346 |
| ≤140 | 138 | 149 | 60.2 vs 47.0 | 0.57 (0.39-0.84) | 0.004 | >140 | 49 | 47 | 59.6 vs 50.7 | 0.84 (0.43-1.63) | 0.596 | 0.377 |
| ≤150 | 146 | 154 | 61.4 vs 47.3 | 0.56 (0.38-0.81) | 0.002 | >150 | 41 | 42 | 54.2 vs 49.9 | 0.92 (0.45-1.87) | 0.822 | 0.218 |
| ≤180 | 159 | 168 | 60.7 vs 50.0 | 0.62 (0.43-0.88) | 0.008 | >180 | 28 | 28 | 54.8 vs 38.9 | 0.64 (0.27-1.47) | 0.289 | 0.948 |
| **Overall survival (OS)** | | | | | | | | | | | | |
| 0 | 54 | 45 | 42.2 vs 37.1 | 0.75 (0.44-1.27) | 0.288 | >0 | 133 | 151 | 49.7 vs 42.1 | 0.76 (0.54-1.07) | 0.119 | 0.911 |
| ≤10 | 67 | 58 | 49.2 vs 38.1 | 0.63 (0.39-1.02) | 0.06 | >10 | 120 | 138 | 46.0 vs 42.1 | 0.85 (0.59-1.21) | 0.365 | 0.404 |
| ≤20 | 81 | 69 | 46.9 vs 36.2 | 0.64 (0.42-0.99) | 0.043 | >20 | 106 | 127 | 47.6 vs 43.6 | 0.87 (0.59-1.27) | 0.465 | 0.343 |
| ≤30 | 88 | 81 | 45.8 vs 38.5 | 0.68 (0.45-1.03) | 0.069 | >30 | 99 | 115 | 48.6 vs 42.3 | 0.84 (0.56-1.25) | 0.382 | 0.547 |
| **≤40** | **94** | **99** | **47.1 vs 39.0** | **0.69 (0.47-1.01)** | **0.058** | **>40** | **93** | **97** | **46.7 vs 42.6** | **0.87 (0.57-1.34)** | **0.527** | **0.459** |
| ≤60 | 108 | 118 | 46.8 vs 38.2 | 0.71 (0.49-1.02) | 0.063 | >60 | 79 | 78 | 46.6 vs 45.9 | 0.87 (0.54-1.41) | 0.579 | 0.517 |
| ≤90 | 125 | 138 | 50.5 vs 39.2 | 0.64 (0.45-0.91) | 0.013 | >90 | 62 | 58 | 37.1 vs 45.5 | 1.15 (0.68-1.93) | 0.61 | 0.071 |
| ≤140 | 138 | 149 | 50.4 vs 37.2 | 0.64 (0.46-0.89) | 0.008 | >140 | 49 | 47 | 32.8 vs 55.5 | 1.42 (0.77-2.61) | 0.258 | 0.022 |
| ≤150 | 146 | 154 | 51.2 vs 37.6 | 0.63 (0.46-0.88) | 0.006 | >150 | 41 | 42 | 25.2 vs 56.3 | 1.60 (0.84-3.05) | 0.154 | 0.009 |
| ≤180 | 159 | 168 | 49.5 vs 40.1 | 0.71 (0.52-0.97) | 0.032 | >180 | 28 | 28 | 28.4 vs 44.8 | 1.15 (0.55-2.42) | 0.716 | 0.197 |

*Univariate Cox regression analysis. Results at the median cutpoint are highlighted in bold.

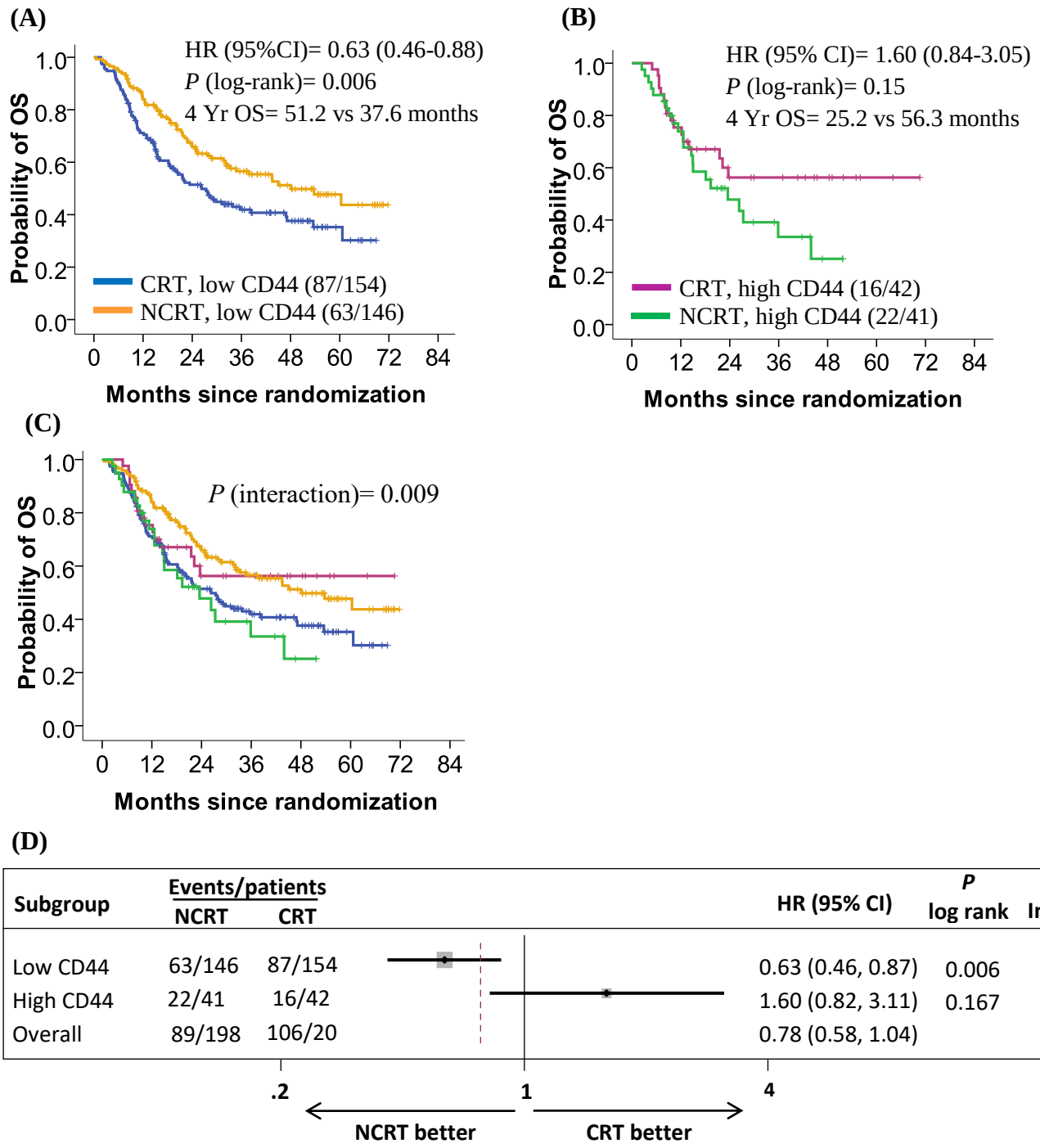



***Figure 36: CD44 status (dichotomized at HScore of 150) showing qualitative interaction with treatments.*** *Kaplan–Meier plots showing overall survival (OS) according to CD44 status (A-C). Forest plot showing bootstrap resampling results (D). A hazard ratio (HR) of less than 1 indicates a benefit with the addition of nimotuzumab; dotted line represents the respective hazard ratio for the overall study population.*

The bootstrap resampling validation confirmed the predictive value of CD44 (dichotomized at HScore 150) for OS [*P* (interaction)=0.015, c index (95%CI)=0.57 (0.53-0.61), Figure 36D]. Low CD44 expression was significantly associated with longer OS in NCRT treated patients relative to CRT [HR (95%CI)= 0.63 (0.46-0.88), *P*=0.006;]. We did not observe similar improvement with NCRT versus CRT in CD44 high subgroup [HR (95%CI)= 1.60 (0.82-3.11), *P*=0.167]

### 5.5.2. Predictive significance of CD44v6 complete membrane expression

Hazard ratios for disease progression, loco-regional failure, and death were statistically significantly less than 1.0 regardless of the cutpoint used to define low CD44v6 expression, suggesting treatment benefit from NCRT compared to CRT (Table 33). Hazard ratios for disease progression and loco-regional failure were less than 1.0 for patients expressing high CD44v6 suggesting treatment benefit from NCRT, although it was statistically significant only at lower cutpoints. Also, the hazard ratios for death were not significant at any of the studied cutpoints. We observed statistically significant qualitative interaction between CD44v6 status dichotomized at cutpoint of 40 and treatment effect for LRC (*P*=0.023, Figure 37) and OS (*P*=0.036, Figure 38) but not for PFS (*P*=0.075).

To confirm predictive impact of CD44v6 for LRC and OS, bootstrap resampling method was used. Predictive impact of CD44v6 for LRC did not show significance in bootstrap resampling validation [P (interaction)= 0.152]. At low CD44v6 expression NCRT did not show significantly improved LRC compared to CRT [HR (95%CI)= 0.25 (0.003-23.5), *P*=0.546].

***Table 33: Cutpoint analysis to assess the predictive role of CD44v6 (complete membrane) expression HScore***

| CD44v6 low | | | | | | CD44v6 high | | | | | | *P* Interaction |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cut point | NCRT (n) | CRT (n) | 4 YR survival (months) | HR (95%CI) (NCRT VS CRT) | *P** | cut point | NCRT (n) | CRT (n) | 4 YR survival (months) | HR (95%CI) (NCRT VS CRT) | *P** | |
| **Progression free survival** | | | | | | | | | | | | |
| 0 | 12 | 18 | 53.3 vs 56.0 | 0.86 (0.28-2.64) | 0.795 | >0 | 184 | 183 | 52.9 vs 39.9 | 0.65 (0.48-0.87) | 0.005 | 0.682 |
| ≤20 | 21 | 32 | 65.4 vs 39.5 | 0.38 (0.15-0.96) | 0.04 | >20 | 175 | 169 | 51.2 vs 41.8 | 0.71 (0.52-0.96) | 0.028 | 0.192 |
| ≤40 | 33 | 40 | 65.4 vs 34.3 | 0.35 (0.17-0.74) | 0.006 | >40 | 163 | 161 | 49.9 vs 43.2 | 0.75 (0.55-1.03) | 0.079 | 0.075 |
| ≤60 | 50 | 56 | 62.6 vs 40.5 | 0.52 (0.28-0.95) | 0.034 | >60 | 146 | 145 | 48.9 vs 41.5 | 0.71 (0.51-0.99) | 0.042 | 0.426 |
| ≤90 | 63 | 68 | 56.6 vs 44.7 | 0.72 (0.43-1.21) | 0.213 | >90 | 133 | 133 | 50.3 vs 39.4 | 0.63 (0.44-0.90) | 0.011 | 0.69 |
| ≤120 | 79 | 90 | 57.3 vs 41.5 | 0.60 (0.38-0.94) | 0.027 | >120 | 117 | 111 | 50.1 vs 40.6 | 0.71 (0.48-1.03) | 0.074 | 0.547 |
| ≤150 | 87 | 98 | 56.5 vs 40.1 | 0.58 (0.37-0.90) | 0.014 | >150 | 109 | 103 | 50.8 vs 41.8 | 0.73 (0.49-1.09) | 0.12 | 0.438 |
| **≤180** | **107** | **111** | **55.4 vs 38.1** | **0.56 (0.38-0.84)** | **0.004** | **>180** | **89** | **90** | **50.9 vs 44.7** | **0.79 (0.51-1.23)** | **0.295** | **0.252** |
| ≤210 | 126 | 124 | 53.3 vs 40.2 | 0.63 (0.44-0.91) | 0.012 | >210 | 70 | 77 | 53.3 vs 41.4 | 0.72 (0.44-1.17) | 0.183 | 0.696 |
| ≤240 | 149 | 150 | 49.3 vs 41.7 | 0.76 (0.55-1.06) | 0.106 | >240 | 47 | 51 | 65.4 vs 41.8 | 0.37 (0.19-0.72) | 0.004 | 0.068 |
| **Loco regional control (LRC)** | | | | | | | | | | | | |
| 0 | 12 | 18 | 65.5 vs 68.2 | 0.66 (0.16-2.62) | 0.549 | >0 | 184 | 183 | 59.5 vs 47.2 | 0.64 (0.46-0.89) | 0.008 | 0.947 |
| ≤20 | 21 | 32 | 80.0 vs 51.6 | 0.24 (0.07-0.85) | 0.026 | >20 | 175 | 169 | 56.9 vs 48.6 | 0.71 (0.50-0.99) | 0.044 | 0.086 |
| ≤40 | 33 | 40 | 77.4 vs 42.9 | 0.25 (0.10-0.62) | 0.003 | >40 | 163 | 161 | 55.5 vs 50.6 | 0.77 (0.54-1.09) | 0.145 | **0.023** |
| ≤60 | 50 | 56 | 70.7 vs 48.3 | 0.47 (0.24-0.93) | 0.029 | >60 | 146 | 145 | 55.2 vs 49.1 | 0.71 (0.49-1.02) | 0.064 | 0.326 |
| ≤90 | 63 | 68 | 65.7 vs 52.3 | 0.66 (0.37-1.19) | 0.168 | >90 | 133 | 133 | 55.9 vs 47.1 | 0.64 (0.43-0.94) | 0.021 | 0.929 |
| ≤120 | 79 | 90 | 65.7 vs 49.1 | 0.54 (0.33-0.91) | 0.021 | >120 | 117 | 111 | 56.0 vs 48.3 | 0.72 (0.47-1.09) | 0.121 | 0.376 |
| ≤150 | 87 | 98 | 64.9 vs 46.8 | 0.51 (0.31-0.84) | 0.007 | >150 | 109 | 103 | 56.6 vs 50.2 | 0.77 (0.50-1.19) | 0.239 | 0.212 |
| **≤180** | **107** | **111** | **65.0 vs 46.3** | **0.50 (0.32-0.78)** | **0.002** | **>180** | **89** | **90** | **53.8 vs 51.4** | **0.87 (0.54-1.40)** | **0.573** | **0.086** |
| ≤210 | 126 | 124 | 61.1 vs 48.2 | 0.60 (0.40-0.89) | 0.012 | >210 | 70 | 77 | 57.2 vs 48.0 | 0.74 (0.44-1.27) | 0.280 | 0.541 |
| ≤240 | 149 | 150 | 57.9 vs 48.8 | 0.70 (0.48-1.0) | 0.049 | >240 | 47 | 51 | 65.4 vs 51.5 | 0.48 (0.24-0.99) | 0.046 | 0.397 |
| **Overall survival (OS)** | | | | | | | | | | | | |
| 0 | 12 | 18 | 41.7 vs 52.5 | 0.85 (0.32-2.30) | 0.755 | >0 | 184 | 183 | 48.5 vs 39.9 | 0.75 (0.56-1.0) | 0.052 | 0.814 |
| ≤20 | 21 | 32 | 54.7 vs 38.5 | 0.49 (0.22-1.08) | 0.077 | >20 | 175 | 169 | 47.0 vs 42.2 | 0.80 (0.59-1.09) | 0.163 | 0.267 |
| ≤40 | 33 | 40 | 56.9 vs 32.2 | 0.38 (0.19-0.74) | 0.004 | >40 | 163 | 161 | 45.8 vs 44.2 | 0.88 (0.64-1.21) | 0.442 | **0.036** |
| ≤60 | 50 | 56 | 53.3 vs 36.3 | 0.58 (0.34-1.01) | 0.054 | >60 | 146 | 145 | 45.2 vs 43.4 | 0.83 (0.59-1.16) | 0.268 | 0.318 |
| ≤90 | 63 | 68 | 48.0 vs 43.6 | 0.82 (0.51-1.34) | 0.43 | >90 | 133 | 133 | 46.9 vs 39.8 | 0.72 (0.51-1.02) | 0.066 | 0.642 |
| ≤120 | 79 | 90 | 52.3 vs 39.4 | 0.66 (0.43-1.0) | 0.052 | >120 | 117 | 111 | 41.9 vs 42.2 | 0.86 (0.59-1.26) | 0.431 | 0.348 |
| ≤150 | 87 | 98 | 53.1 vs 37.5 | 0.63 (0.42-0.94) | 0.024 | >150 | 109 | 103 | 40.2 vs 44.7 | 0.91 (0.61-1.36) | 0.656 | 0.198 |
| **≤180** | **107** | **111** | **52.7 vs 37.7** | **0.61 (0.42-0.89)** | **0.01** | **>180** | **89** | **90** | **36.4 vs 45.4** | **1.03 (0.67-1.60)** | **0.886** | **0.081** |
| ≤210 | 126 | 124 | 52.9 vs 38.2 | 0.63 (0.44-0.89) | 0.01 | >210 | 70 | 77 | 31.1 vs 47.8 | 1.10 (0.68-1.78) | 0.688 | 0.074 |
| ≤240 | 149 | 150 | 48.0 vs 41.9 | 0.78 (0.57-1.08) | 0.133 | >240 | 47 | 51 | 38.6 vs 40.2 | 0.66 (0.36-1.22) | 0.182 | 0.633 |

*Univariate Cox regression analysis. Results at the median cutpoint are highlighted in bold.

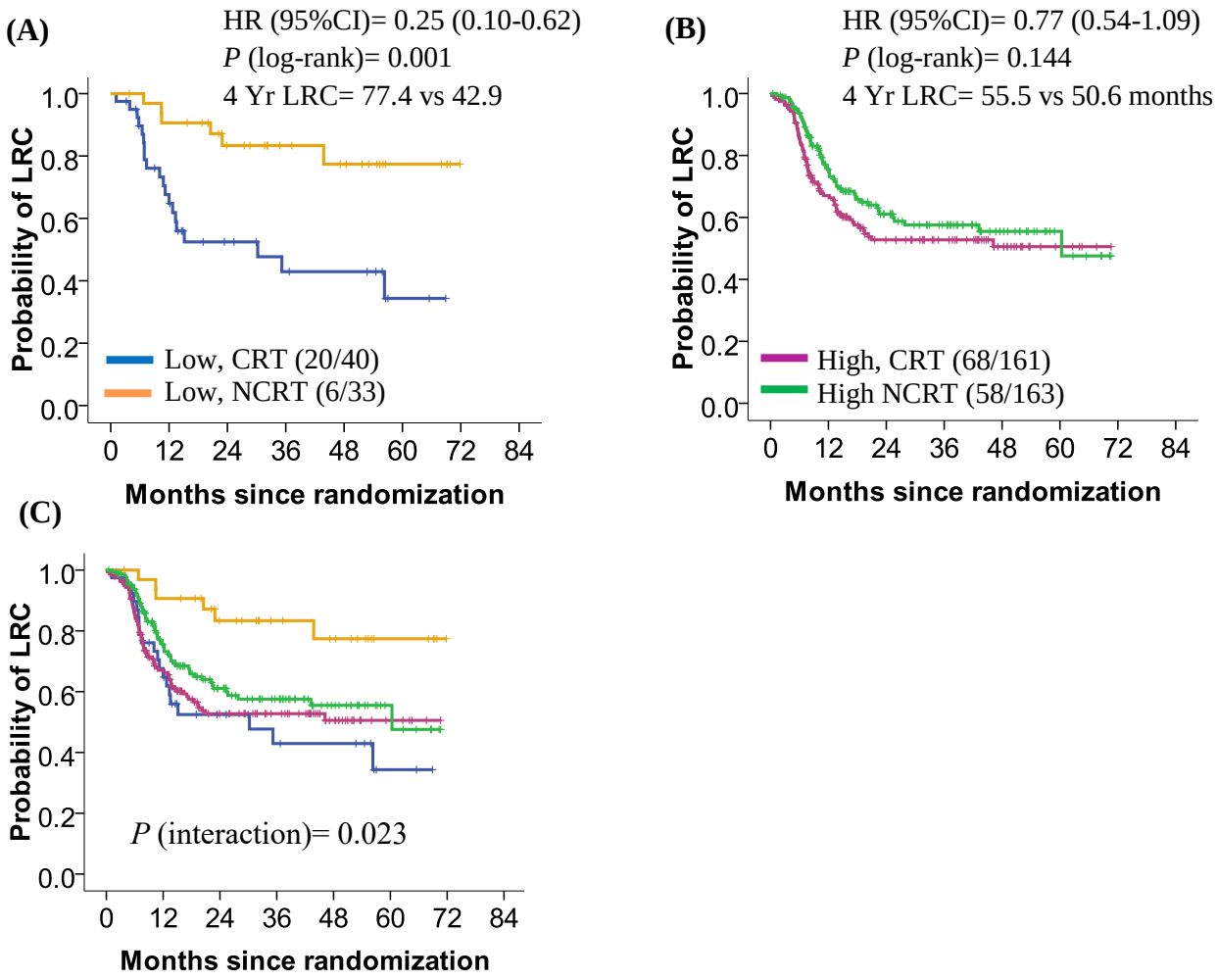



***Figure 37: CD44v6 status (dichotomized at HScore of 40) showing qualitative interaction with treatments.*** *Kaplan–Meier plots showing LRC for LA-HNSCC patients according to CD44v6 expression status and treatment group (A-C)*

Predictive value of CD44v6 status dichotomized at HScore=40 for OS was confirmed by bootstrap resampling [*P* (interaction)=0.041, c index (95%CI)= 0.56 (0.52-0.60]. Wherein at low CD44v6 expression NCRT performed significantly better compared to CRT [HR (95%CI)= 0.38 (0.19-0.76), *P*=0.007] and no significant difference in OS was observed in patients with high CD44v6 expression [HR (95%CI)= 0.88 (0.64-1.22), *P*=0.455, Figure 38D].

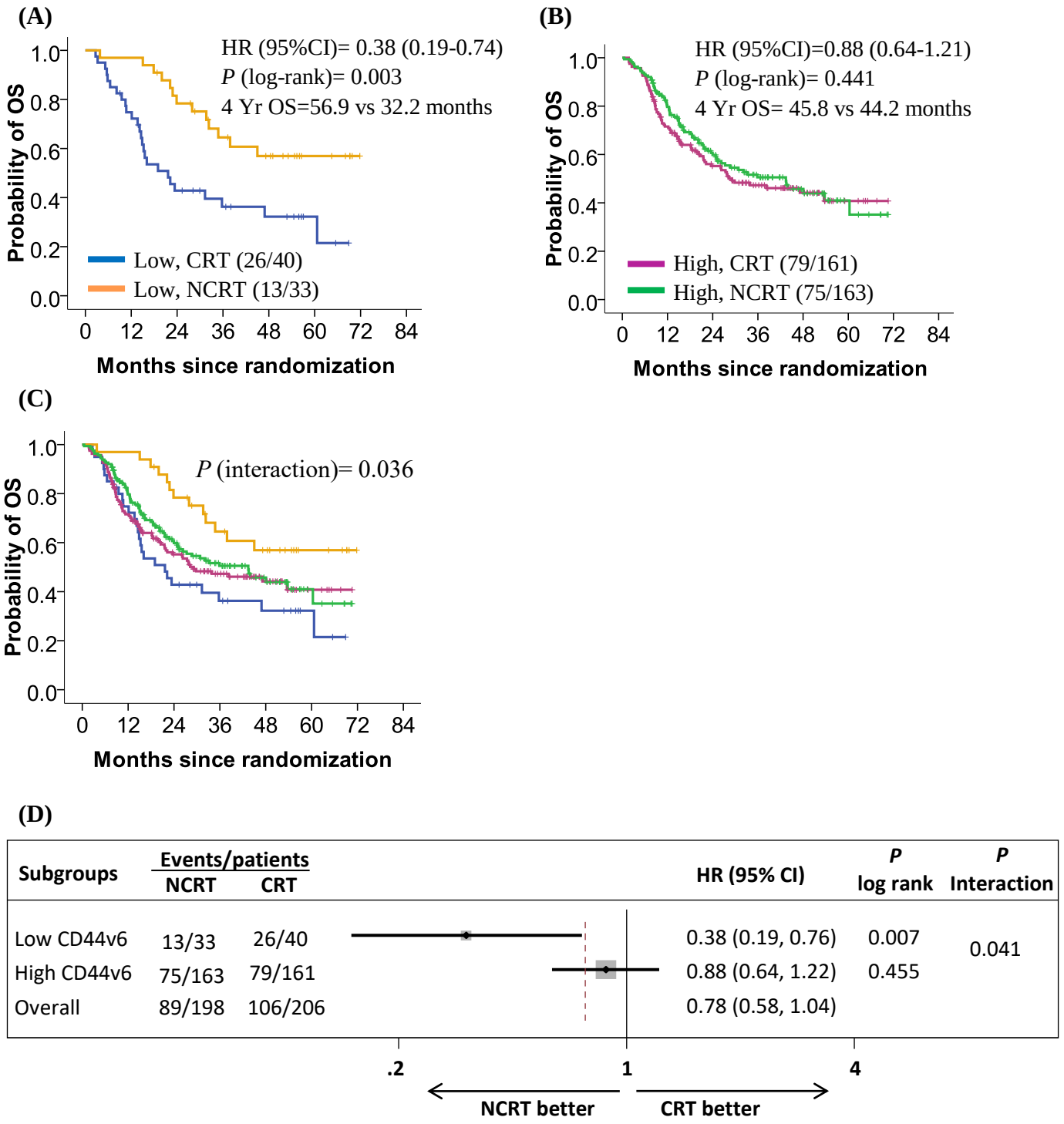



***Figure 38: CD44v6 status (dichotomized at HScore of 40) showing qualitative interaction with treatments.*** *Kaplan–Meier plots showing OS according to CD44v6 expression status (A-C); Forest plot showing bootstrap resampling results (D). A hazard ratio (HR) of less than 1 indicates a benefit from NCRT; dotted line represents hazard ratio for the overall study population.*

### 5.5.3. Predictive significance of CD98hc, ALDH1A1 and SOX2 expression

We analyzed the predictive impact of complete membrane expression of CD98hc at different possible cutpoints. Overall, the hazard ratios for disease progression, loco-regional failure, and death were less than 1.0 regardless of the cutpoint used to define low or high CD98hc complete membrane expression (Table 34). No statistically significant interaction between CD98hc status and treatment effect was observed at any of the studied cutpoints, suggesting that treatment benefits from NCRT relative to CRT are independent of the CD98hc expression status.

We next analyzed cytoplasmic ALDH1A1 expression dichotomized at different possible cutpoints to find its predictive association. Interestingly, the hazard ratios for disease progression, loco-regional failure, and death were significantly less than 1.0 irrespective of the cutpoints used to define low ALDH1A1 expression, suggesting treatment benefit from NCRT relative to CRT (Table 35). No statistically significant differences in the PFS, LRC, or OS were observed between two treatments at any of the cutpoints used for defining high ALDH1A1. However, we did not find a statistically significant interaction between ALDH1A1 status and treatment effect at any of the studied cutpoints for any of the clinical endpoint which might be due to the small sample size of the subgroups with a high ALDH1A1 expression as more than 45% of the samples were negative for ALDH1A1 expression. Therefore, the predictive value of this biomarker needs to be studied in a larger cohort of HNSCC patients.

Hazard ratios for disease progression, loco-regional failure, and death of patients treated with NCRT relative to CRT treated patients for patients with tumors expressing low or high nuclear SOX2 dichotomized at different possible cutpoints with corresponding interaction *P* values are displayed in Table 36. The hazard ratios for disease progression, loco-regional failure, and death were less than 1.0 for low or high SOX2 expressing patients suggesting treatment benefit from

NCRT relative to CRT. We did not observe any significant interaction between SOX2 status and treatment effect, suggesting that SOX2 expression status is not predictive of improved treatment response to NCRT.

***Table 34: Cutpoint analysis to assess the predictive role of CD98hc (complete membrane) expression HScore***

| CD98hc low | | | | | | CD98hc high | | | | | | *P* Interaction |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cut point | NCRT (n) | CRT (n) | 4 YR survival (months) | HR (95%CI) (NCRT VS CRT) | *P** | cut point | NCRT (n) | CRT (n) | 4 YR survival (months) | HR (95%CI) (NCRT VS CRT) | *P** | |
| **Progression free survival** | | | | | | | | | | | | |
| 0 | 49 | 35 | 63.1 vs 62.8 | 0.74 (0.35-1.59) | 0.44 | >0 | 133 | 153 | 49.0 vs 37.5 | 0.68 (0.48-0.94) | 0.022 | 0.818 |
| ≤20 | 60 | 52 | 59.7 vs 56.6 | 0.69 (0.38-1.25) | 0.221 | >20 | 122 | 136 | 49.2 vs 36.2 | 0.66 (0.46-0.94) | 0.023 | 0.913 |
| ≤40 | 80 | 77 | 59.0 vs 49.7 | 0.62 (0.38-1.01) | 0.057 | >40 | 102 | 111 | 48.1 vs 36.3 | 0.69 (0.47-1.01) | 0.058 | 0.723 |
| **≤60** | **96** | **99** | **54.3 vs 46.6** | **0.64 (0.42-0.99)** | **0.044** | **>60** | **86** | **89** | **51.2 vs 36.7** | **0.67 (0.43-1.03)** | **0.069** | **0.884** |
| ≤90 | 117 | 118 | 51.1 vs 44.6 | 0.70 (0.48-1.02) | 0.066 | >90 | 65 | 70 | 57.0 vs 37.6 | 0.57 (0.34-0.96) | 0.033 | 0.584 |
| ≤120 | 134 | 136 | 51.3 vs 44.9 | 0.71 (0.50-1.01) | 0.058 | >120 | 48 | 52 | 57.4 vs 34.3 | 0.51 (0.28-0.93) | 0.028 | 0.409 |
| ≤180 | 163 | 158 | 52.3 vs 43.2 | 0.68 (0.49-0.94) | 0.02 | >180 | 19 | 30 | 59.1 vs 33.8 | 0.46 (0.18-1.18) | 0.104 | 0.505 |
| **Loco-regional control (LRC)** | | | | | | | | | | | | |
| 0 | 49 | 35 | 69.9 vs 77.4 | 1.05 (0.41-2.66) | 0.922 | >0 | 133 | 153 | 55.4 vs 44.6 | 0.66 (0.45-0.95) | 0.024 | 0.354 |
| ≤20 | 60 | 52 | 68.2 vs 67.3 | 0.73 (0.36-1.46) | 0.371 | >20 | 122 | 136 | 54.6 vs 43.8 | 0.66 (0.45-0.98) | 0.037 | 0.812 |
| ≤40 | 80 | 77 | 68.2 vs 59.6 | 0.58 (0.32-1.03) | 0.063 | >40 | 102 | 111 | 52.7 vs 43.8 | 0.72 (0.48-1.09) | 0.122 | 0.539 |
| **≤60** | **96** | **99** | **63.3 vs 55.7** | **0.59 (0.36-0.97)** | **0.037** | **>60** | **86** | **89** | **55.0 vs 44.5** | **0.73 (0.46-1.16)** | **0.18** | **0.547** |
| ≤90 | 117 | 118 | 59.0 vs 54.2 | 0.68 (0.44-1.05) | 0.08 | >90 | 65 | 70 | 61.0 vs 44.2 | 0.62 (0.36-1.07) | 0.087 | 0.824 |
| ≤120 | 134 | 136 | 58.8 vs 53.8 | 0.69 (0.46-1.03) | 0.069 | >120 | 48 | 52 | 61.2 vs 41.4 | 0.58 (0.31-1.09) | 0.091 | 0.672 |
| ≤180 | 163 | 158 | 59.6 vs 51.0 | 0.67 (0.47-0.96) | 0.03 | >180 | 19 | 30 | 59.1 vs 45.7 | 0.61 (0.23-1.61) | 0.315 | 0.863 |
| **Overall survival (OS)** | | | | | | | | | | | | |
| 0 | 49 | 35 | 62.1 vs 63.0 | 0.83 (0.39-1.73) | 0.613 | >0 | 133 | 153 | 44.7 vs 38.1 | 0.76 (0.54-1.05) | 0.094 | 0.858 |
| ≤20 | 60 | 52 | 59.9 vs 61.3 | 0.75 (0.41-1.36) | 0.336 | >20 | 122 | 136 | 44.0 vs 35.1 | 0.76 (0.54-1.07) | 0.119 | 0.919 |
| ≤40 | 80 | 77 | 60.2 vs 53.9 | 0.74 (0.45-1.22) | 0.237 | >40 | 102 | 111 | 40.5 vs 33.4 | 0.74 (0.51-1.07) | 0.106 | 0.983 |
| **≤60** | **96** | **99** | **56.4 vs 50.0** | **0.71 (0.46-1.10)** | **0.129** | **>60** | **86** | **89** | **42.2 vs 33.7** | **0.75 (0.50-1.13)** | **0.163** | **0.894** |
| ≤90 | 117 | 118 | 51.7 vs 47.5 | 0.85 (0.58-1.24) | 0.393 | >90 | 65 | 70 | 46.2 vs 32.9 | 0.58 (0.36-0.94) | 0.026 | 0.232 |
| ≤120 | 134 | 136 | 52.1 vs 45.9 | 0.80 (0.56-1.15) | 0.232 | >120 | 48 | 52 | 45.1 vs 32.4 | 0.57 (0.33-0.99) | 0.044 | 0.312 |
| ≤180 | 163 | 158 | 50.2 vs 45.3 | 0.78 (0.57-1.08) | 0.136 | >180 | 19 | 30 | 46.1 vs 25.3 | 0.50 (0.21-1.18) | 0.114 | 0.362 |

*Univariate Cox regression analysis. Results at the median cutpoint are highlighted in bold.

***Table 35: Cutpoint analysis to assess the predictive role of cytoplasmic ALDH1A1 expression HScore***

| | ALDH1A1 low | | | | | ALDH1A1 high | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **cut point** | **NCRT (n)** | **CRT (n)** | **4 YR survival (months)** | **HR (95%CI) (NCRT VS CRT)** | ***P**** | **cut point** | **NCRT (n)** | **CRT (n)** | **4 YR survival (months)** | **HR (95%CI) (NCRT VS CRT)** | ***P**** | ***P* Interaction** |
| **Progression free survival** | | | | | | | | | | | | |
| **≤5** | **86** | **97** | **58.4 vs 48.9** | **0.70 (0.45-1.09)** | **0.115** | **>5** | **91** | **84** | **47.8 vs 34.8** | **0.63 (0.41-0.98)** | **0.04** | **0.809** |
| ≤20 | 102 | 116 | 57.4 vs 43.9 | 0.63 (0.42-0.94) | 0.024 | >20 | 75 | 65 | 47.6 vs 41.0 | 0.76 (0.46-1.24) | 0.266 | 0.58 |
| ≤40 | 124 | 130 | 59.1 vs 45.5 | 0.61 (0.42-0.89) | 0.011 | >40 | 53 | 51 | 39.2 vs 35.1 | 0.84 (0.49-1.45) | 0.525 | 0.36 |
| ≤70 | 146 | 138 | 57.8 vs 43.4 | 0.60 (0.42-0.86) | 0.005 | >70 | 31 | 43 | 30.5 vs 40.3 | 1.08 (0.56-2.08) | 0.817 | 0.142 |
| ≤100 | 151 | 142 | 56.0 vs 42.8 | 0.62 (0.44-0.87) | 0.006 | >100 | 26 | 39 | 39.0 vs 41.2 | 1.02 (0.49-2.13) | 0.953 | 0.245 |
| ≤140 | 159 | 152 | 54.3 vs 44.0 | 0.67 (0.48-0.93) | 0.017 | >140 | 18 | 29 | 53.1 vs 32.2 | 0.72 (0.29-1.80) | 0.483 | 0.891 |
| ≤160 | 164 | 165 | 54.3 vs 42.2 | 0.66 (0.48-0.91) | 0.012 | >160 | 13 | 16 | NA vs 53.5 | 0.82 (0.26-2.57) | 0.727 | 0.691 |
| **Loco-regional control (LRC)** | | | | | | | | | | | | |
| **≤5** | **86** | **97** | **65.8 vs 58.3** | **0.69 (0.42-1.13)** | **0.14** | **>5** | **91** | **84** | **54.7 vs 41.7** | **0.65 (0.40-1.04)** | **0.073** | **0.889** |
| ≤20 | 102 | 116 | 64.5 vs 54.1 | 0.63 (0.40-1.0) | 0.047 | >20 | 75 | 65 | 55.0 vs 45.7 | 0.74 (0.43-1.26) | 0.265 | 0.683 |
| ≤40 | 124 | 130 | 64.8 vs 54.6 | 0.62 (0.41-0.95) | 0.028 | >40 | 53 | 51 | 50.0 vs 40.7 | 0.81 (0.44-1.47) | 0.485 | 0.508 |
| ≤70 | 146 | 138 | 65.3 vs 52.0 | 0.58 (0.39-0.86) | 0.006 | >70 | 31 | 43 | 33.9 vs 47.0 | 1.29 (0.63-2.62) | 0.489 | 0.069 |
| ≤100 | 151 | 142 | 63.1 vs 51.8 | 0.61 (0.42-0.90) | 0.012 | >100 | 26 | 39 | 44.2 vs 46.3 | 1.09 (0.49-2.44) | 0.827 | 0.233 |
| ≤140 | 159 | 152 | 62.3 vs 53.2 | 0.66 (0.45-0.96) | 0.028 | >140 | 18 | 29 | 48.3 vs 34.4 | 0.78 (0.32-1.88) | 0.576 | 0.73 |
| ≤160 | 164 | 165 | 61.2 vs 50.7 | 0.66 (0.46-0.94) | 0.022 | >160 | 13 | 16 | NA vs 57.9 | 0.81 (0.23-2.9) | 0.75 | 0.689 |
| **Overall survival (OS)** | | | | | | | | | | | | |
| **≤5** | **86** | **97** | **54.5 vs 48.0** | **0.75 (0.48-1.16)** | **0.20** | **>5** | **91** | **84** | **43.9 vs 39.0** | **0.79 (0.51-1.21)** | **0.272** | **0.912** |
| ≤20 | 102 | 116 | 54.5 vs 42.9 | 0.69 (0.46-1.02) | 0.062 | >20 | 75 | 65 | 41.8 vs 47.6 | 0.95 (0.58-1.54) | 0.827 | 0.336 |
| ≤40 | 124 | 130 | 54.6 vs 42.7 | 0.68 (0.47-0.98) | 0.039 | >40 | 53 | 51 | 34.9 vs 49.6 | 1.05 (0.60-1.84) | 0.863 | 0.205 |
| ≤70 | 146 | 138 | 51.3 vs 40.4 | 0.68 (0.48-0.95) | 0.024 | >70 | 31 | 43 | 37.1 vs 58.4 | 1.35 (0.65-2.78) | 0.421 | 0.089 |
| ≤100 | 151 | 142 | 50.2 vs 40.6 | 0.70 (0.50-0.98) | 0.035 | >100 | 26 | 39 | 43.8 vs 59.9 | 1.25 (0.57-2.78) | 0.577 | 0.176 |
| ≤140 | 159 | 152 | 49.6 vs 42.3 | 0.75 (0.54-1.04) | 0.085 | >140 | 18 | 29 | 47.3 vs 55.8 | 0.96 (0.40-2.30) | 0.931 | 0.656 |
| ≤160 | 164 | 165 | 50.5 vs 42.5 | 0.74 (0.54-1.02) | 0.067 | >160 | 13 | 16 | 23.8 vs 61.9 | 1.28 (0.41-4.05) | 0.674 | 0.345 |

*Univariate Cox regression analysis. Results at the median cutpoint are highlighted in bold.

***Table 36: Cutpoint analysis to assess the predictive role of nuclear SOX2 expression HScore***

| SOX2 low | | | | | | SOX2 high | | | | | | *P* Interaction |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cut point | NCRT (n) | CRT (n) | 4 YR survival (months) | HR (95%CI) (NCRT VS CRT) | *P** | cut point | NCRT (n) | CRT (n) | 4 YR survival (months) | HR (95%CI) (NCRT VS CRT) | *P** | |
| **Progression free survival** | | | | | | | | | | | | |
| 0 | 44 | 47 | 62.4 vs 43.1 | 0.50 (0.26-0.95) | 0.034 | >0 | 125 | 129 | 52.0 vs 40.0 | 0.68 (0.47-0.98) | 0.038 | 0.364 |
| ≤20 | 70 | 79 | 58.4 vs 44.3 | 0.61 (0.37-1.0) | 0.05 | >20 | 99 | 97 | 52.5 vs 37.5 | 0.64 (0.42-0.97) | 0.034 | 0.808 |
| **≤40** | **86** | **95** | **59.3 vs 42.7** | **0.58 (0.37-0.91)** | **0.017** | **>40** | **83** | **81** | **49.8 vs 38.5** | **0.69 (0.44-1.10)** | **0.11** | **0.499** |
| ≤70 | 98 | 110 | 55.7 vs 42.0 | 0.65 (0.44-0.98) | 0.038 | >70 | 71 | 66 | 53.2 vs 39.4 | 0.60 (0.36-1.01) | 0.056 | 0.882 |
| ≤100 | 113 | 123 | 56.6 vs 40.9 | 0.59 (0.40-0.87) | 0.007 | >100 | 56 | 53 | 52.7 vs 42.0 | 0.74 (0.41-1.32) | 0.301 | 0.491 |
| ≤140 | 127 | 144 | 54.0 vs 39.6 | 0.57 (0.40-0.82) | 0.002 | >140 | 42 | 32 | 44.3 vs 56.3 | 0.95 (0.47-1.94) | 0.89 | 0.21 |
| ≤210 | 151 | 151 | 56.3 vs 41.1 | 0.59 (0.42-0.83) | 0.002 | >210 | 18 | 25 | 45.7 vs 36.3 | 1.0 (0.40-2.48) | 0.994 | 0.276 |
| **Loco-regional control (LRC)** | | | | | | | | | | | | |
| 0 | 44 | 47 | 64.2 vs 55.6 | 0.64 (0.32-1.27) | 0.2 | >0 | 125 | 129 | 62.0 vs 47.9 | 0.61 (0.40-0.93) | 0.02 | 0.959 |
| ≤20 | 70 | 79 | 64.4 vs 55.8 | 0.69 (0.40-1.20) | 0.188 | >20 | 99 | 97 | 61.4 vs 44.2 | 0.56 (0.35-0.90) | 0.017 | 0.671 |
| **≤40** | **86** | **95** | **65.4 vs 56.3** | **0.68 (0.41-1.13)** | **0.134** | **>40** | **83** | **81** | **59.3 vs 42.1** | **0.56 (0.34-0.93)** | **0.026** | **0.717** |
| ≤70 | 98 | 110 | 65.5 vs 53.3 | 0.64 (0.41-1.02) | 0.06 | >70 | 71 | 66 | 57.8 vs 44.1 | 0.58 (0.33-1.01) | 0.056 | 0.834 |
| ≤100 | 113 | 123 | 65.0 vs 51.3 | 0.59 (0.38-0.91) | 0.017 | >100 | 56 | 53 | 58.7 vs 47.0 | 0.68 (0.36-1.30) | 0.243 | 0.672 |
| ≤140 | 127 | 144 | 65.2 vs 49.8 | 0.57 (0.38-0.86) | 0.007 | >140 | 42 | 32 | 54.8 vs 42.0 | 0.86 (0.39-1.93) | 0.717 | 0.378 |
| ≤210 | 151 | 151 | 63.0 vs 50.9 | 0.62 (0.42-0.90) | 0.012 | >210 | 18 | 25 | 63.5 vs 39.3 | 0.57 (0.18-1.81) | 0.336 | 0.916 |
| **Overall survival (OS)** | | | | | | | | | | | | |
| 0 | 44 | 47 | 60.9 vs 36.6 | 0.49 (0.27-0.91) | 0.024 | >0 | 125 | 129 | 48.7 vs 43.4 | 0.83 (0.58-1.19) | 0.308 | 0.133 |
| ≤20 | 70 | 79 | 58.0 vs 43.3 | 0.61 (0.38-0.99) | 0.044 | >20 | 99 | 97 | 47.0 vs 39.4 | 0.81 (0.54-1.22) | 0.32 | 0.351 |
| **≤40** | **86** | **95** | **55.8 vs 41.2** | **0.64 (0.42-0.99)** | **0.045** | **>40** | **83** | **81** | **46.5 vs 42.4** | **0.83 (0.53-1.31)** | **0.434** | **0.396** |
| ≤70 | 98 | 110 | 52.0 vs 40.8 | 0.72 (0.49-1.07) | 0.11 | >70 | 71 | 66 | 52.1 vs 45.0 | 0.72 (0.43-1.19) | 0.2 | 0.945 |
| ≤100 | 113 | 123 | 53.3 vs 40.8 | 0.68 (0.47-0.99) | 0.046 | >100 | 56 | 53 | 49.1 vs 45.1 | 0.79 (0.45-1.39) | 0.413 | 0.694 |
| ≤140 | 127 | 144 | 54.0 vs 39.6 | 0.65 (0.46-0.93) | 0.017 | >140 | 42 | 32 | 44.3 vs 56.3 | 1.08 (0.52-2.26) | 0.829 | 0.234 |
| ≤210 | 151 | 151 | 52.0 vs 39.6 | 0.69 (0.49-0.95) | 0.024 | >210 | 18 | 25 | 51.6 vs 58.9 | 1.03 (0.38-2.78) | 0.95 | 0.494 |

*Univariate Cox regression analysis. Results at the median cutpoint are highlighted in bold.

## 5.6. Summary and Discussion

Here we present analyses of the prognostic and predictive role of different putative CSC markers in HPV negative LA-HNSCC patients treated in a randomized clinical trial with either CRT or NCRT. We have demonstrated that the complete membrane expression of CD44 and CD44v6 has the potential for predicting NCRT treatment response. In addition, we also showed an independent prognostic role of CD98hc complete membrane expression. The prognostic significance of CD98hc in HNSCC is reported in the literature, however, this is the first study demonstrating the predictive role of CD44 and CD44v6 in HPV negative LA-HNSCC patients for anti-EGFR based treatment response.

In HNSCCs, CD44 and its variant isoforms are studied widely for their prognostic role; however, the reports are majorly conflicting (115). Additionally, there is limited literature on the predictive role of CD44 and CD44v6 expression for EGFR treatment response in HNSCCs. Therefore, we have evaluated both prognostic and predictive role of complete membrane expression of CD44 and CD44v6 in phase 3 randomized setting. We did not observe prognostic association of CD44 or CD44v6 membrane expression at any HScore cutpoints. Lack of prognostic association of CD44 has also been reported by Rietbergen et al in HPV DNA negative HNSCC patients (57). Interestingly, CD44 and CD44v6 status showed significant qualitative interaction with treatment effect for OS that remained significant even in the bootstrap validation. The low membrane expression of CD44 or CD44v6 was significantly associated with better treatment response to NCRT relative to CRT. High expression of CD44 or CD44v6 was not associated with similar benefits from NCRT treatment. Preclinical reports in breast cancer, NSCLC, and HNSCC have shown that CD44 and its isoforms interact with ErbB family members and act as a co-receptor for activation of these receptors (24, 89, 90). Downregulation

of CD44 or its isoforms is associated with a decrease in the EGFR signaling, tumor growth, and metastasis (24, 90). Ours is the first report showing the role of CD44 and CD44v6 in predicting EGFR based treatment response in HPV negative HNSCC patients. Our results also indicate that the high expression of CD44 or CD44v6 might be associated with the resistance to EGFR based treatment and thus are a therapeutic target in these patients.

Negative prognostic association of CD98hc (SLC3A2) gene expression in HPV negative LA-HNSCC is reported recently by Linge et al (116). In the current study, we showed that CD98hc expression is an independent negative prognostic biomarker of LRC and OS in HPV negative LA-HNSCC patients. CD98hc. CD98hc, however, did not show any predictive role in these patients. Hypoxia and activation of HIF1α signaling are important features of the tumor microenvironment and known regulating factors of CSC phenotype (117, 118). Interestingly, we also showed that HIF1α expression was a stronger prognosticator of LRC and OS as compared to CD98hc which is a known putative CSC marker in HNSCC (21).

We also analyzed the prognostic and predictive impact of ALDH1A1 and SOX2. ALDH1A1 expression did not show any significant prognostic association at any of the studied HScore cutpoints. However, we observed that the low expression of ALDH1A1 was consistently associated with significantly better clinical response to NCRT compared to CRT at all the analyzed cutpoints. Although, we could not detect any significant interaction between ALDH1A1 status and treatment effects that might be due to the small number of ALDH1A1 positive cases. The predictive role of ALDH1A1 expression, therefore, needs to be studied in a larger cohort. In the literature majority of the reports have shown a negative prognostic impact of SOX2; however contradictory reports also exist showing a positive prognostic impact of SOX2 expression in HNSCCs as well as other cancers (99, 119). However, in the current study, we did

not observe any prognostic or predictive association of SOX2 expression. We did not detect any nuclear expression of OCT4A in any of the tumor tissues; however, we did observe strong nuclear OCT4A expression in testicular seminoma that was used as a positive control in every IHC batch. Mallo et al has also reported similar observations in 348 tumor tissues of HNSCC (28).

There are few limitations of the current study which need to be considered. One of the limitations is the semi-quantitative assessment of IHC staining by pathologists which is inherently subjective. In the future, whole-slide digital image analysis needs to be used for objective assessment of the staining which will allow for cross-study comparisons. In addition use of multiple cutpoints for finding association has a risk of inflation of the type I error and overestimation of the results (120). Therefore, these associations need to be validated in an independent study to test the strength of these observations.

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# 6.

# Results-III

# Correlation of transcriptionally active human papillomavirus status with the clinical and molecular profiles of HNSCC

### 6.1. Study groups and patients' baseline clinical and demographic parameters

We compared the clinical outcomes and expression of different hypoxia, EGFR based and CSC markers in three different patients subgroups- patients with HPV positive HNSCC (RNA-ISH positive, n=25, Figure 6C); HPV negative HNSCC (n=49) matched with HPV positive cases for baseline characteristics (gender, age, N classification, TNM stage, major tumor site, and tobacco habit status) using the propensity score matching method; and p16 positive HNSCC (≥70% tumor cells with nuclear p16 expression and without transcriptionally active HPV; n=20). The demographic details of the three subgroups are provided in Table 37**.** Case-wise details of baseline characteristics and HPV testing results of the patients included in these three groups are tabulated in Appendix Tables 1-3.

***Table 37: Demographic characteristics of HNSCC patients included in the study***

| Characteristics | HPV positive (n=25) | Matched HPV negative (n=49) | p16 positive (n=20) |
|---|---|---|---|
| **Age (Years)** | | | |
| Median (range) | 42 (20-66) | 51 (28-67) | 58 (30-67) |
| 40 or below | 10 (40) | 7 (14.3) | 2 (10) |
| >40 and <60 | 12 (48) | 38 (77.6) | 11 (55) |
| 60 and above | 3 (12) | 4 (8.2) | 7 (35) |
| **Gender** | | | |
| Male | 14 (56) | 32 (65.3) | 16 (80) |
| Female | 11 (44) | 17 (34.7) | 4 (20) |
| **Site of tumor** | | | |
| Hypopharynx | 2 (8) | 7 (14.3) | 8 (40) |
| Larynx | 5 (20) | 6 (12.2) | 4 (20) |
| Oropharynx | 18 (72) | 36 (73.5) | 8 (40) |
| **Clinical stage (AJCC 8th edition)** | | | |
| II | 9 (36) | 0 (0) | 0 (0) |
| III | 9 (36) | 5 (10.2) | 2 (10) |
| IV | 7 (28) | 44 (89.8) | 18 (90) |
| **T stage (AJCC 8th edition)** | | | |
| T1-T2 | 8 (32) | 13 (26.5) | 6 (30) |
| T3-T4 | 17 (68) | 36 (73.5) | 14 (70) |
| **N stage (AJCC 8th edition)** | | | |

| | | | |
|---|---|---|---|
| N0-N1 | 4 (16) | 8 (16.3) | 4 (20) |
| N2-N3 | 21 (84) | 41 (83.7) | 16 (80) |
| **Extra capsular extension** | | | |
| No | 15 (60) | 35 (71.4) | 17 (85) |
| Yes | 10 (40) | 14 (28.6) | 3 (15) |
| **Tobacco and/or alcohol habit profile** | | | |
| No habits | 11 (44) | 13 (26.5) | 1 (5) |
| Exclusive chewer | 4 (16) | 8 (16.3) | 2 (10) |
| Exclusive smoker | 4 (16) | 4 (8.2) | 5 (25) |
| Exclusive drinker | 2 (8) | 0 (0) | 0 (0) |
| Mixed habits | 3 (12) | 24 (49) | 11 (55) |
| No information | 1 (4) | 0 (0) | 1 (5) |
| **Tobacco chewing** | | | |
| Non chewer | 17 (68) | 28 (57.1) | 8 (40) |
| Light (chewing index <80) | 4 (16) | 10 (20.4) | 4 (20) |
| Heavy (chewing index >80) | 3 (12) | 11 (22.5) | 7 (35) |
| No information | 0 (0) | 0 (0) | 0 (0) |
| **Bidi smoking** | | | |
| Non smoker | 19 (76) | 25 (51) | 4 (20) |
| <10 pack-years | 5 (20) | 15 (30.6) | 9 (45) |
| >10 pack-years | 0 (0) | 9 (18.4) | 6 (30) |
| No information | 0 (0) | 0 (0) | 0 (0) |
| **Cigarette smoking** | | | |
| No smoker | 20 (80) | 42 (85.7) | 14 (70) |
| <10 pack-years | 4 (16) | 5 (10.2) | 5 (25) |
| >10 pack-years | 0 (0) | 2 (4.1) | 0 (0) |
| No information | 0 (0) | 0 (0) | 0 (0) |
| **Treatment received** | | | |
| cisplatin-radiation | 12 (48) | 29 (59.1) | 11 (55) |
| cisplatin-radiation plus nimotuzumab | 13 (52) | 20 (40.9) | 9 (45) |

Data are number (%) unless otherwise indicated. p16 positive=high risk HPV RNA-ISH negative but showing moderate to strong diffused nuclear and cytoplasmic p16 staining in ≥70% tumor cells according to CAP (College of American Pathologist) criteria. Tobacco chewing index is calculated as frequency of chewing event per day multiplied by duration in years; A pack-year is defined as the equivalent of smoking one pack of bidis (25 bidis/pack) or one pack of cigarettes (10 cigarettes/pack) per day for 1 year.

### 6.2. Association of HPV status with clinical outcomes in HNSCC patients

Univariate Cox analysis showed that HPV positive OPSCC patients (n=18) exhibited significantly better clinical outcomes than the HPV negative OPSCC patients (n=196), PFS [HR (95%CI) =0.31 (0.11-0.84), 76.4 vs 36.3 months, log rank *P*=0.014, Figure 39A), LRC [HR (95%CI) =0.28 (0.09-0.88), 81.5 vs 43.7 months, log rank *P*=0.02, Figure 39B] and OS [HR (95%CI) =0.31 (0.12-0.85), 70.1 vs 36.5 months, log rank *P*=0.016, Figure 39C].

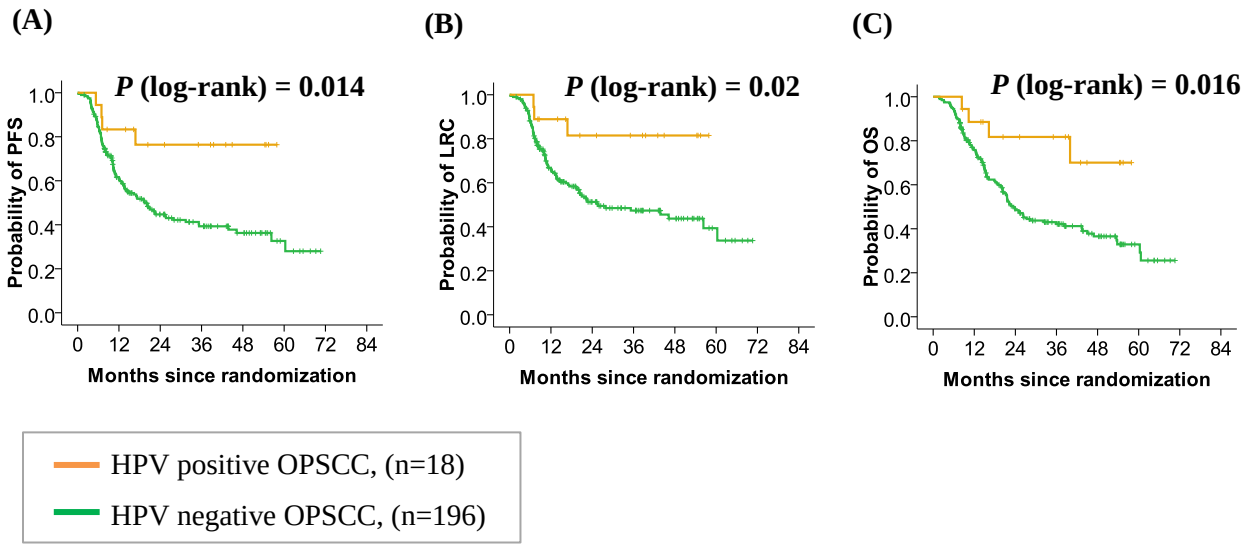


***Figure 39: Kaplan-Meier plots showing clinical outcomes in OPSCC patients according to HPV status.*** *Progression free survival (A), Loco-regional control (B) and Overall survival (C) in OPSCC patients.*

HPV positive patients also showed significantly better outcomes when compared to the matched HPV negative subgroup [PFS: log-rank *P*=0.002, LRC: log-rank *P*=0.001 and OS: log-rank *P*=0.011; Table 38, Figure 40]. The clinical outcomes varied between the p16 positive and HPV positive subgroups. However, the differences were significant only for OS (log-rank *P*=0.021)

and not for PFS (log-rank *P*=0.053) or LRC (log-rank *P*=0.098, Table 38, Figure 40). PFS, LRC, and OS were not significantly different between the HPV negative and p16 positive subgroups (Table 38, Figure 40).

***Table 38: Difference in the clinical outcomes in different study groups characterized by tumor p16 expression and tumor HPV status***

| | **[a]HPV positive (n=25) vs [b]HPV negative (n=49)** | **[c]p16 positive (n=20) vs HPV negative (n=49)** | **HPV positive (n=25) vs p16 positive (n=20)** |
|---|---|---|---|
| **Progression free survival (PFS)** | | | |
| Events (%) | 6 (24) vs 31 (63) | 10 (50) vs 31 (63) | 6 (24) vs 10 (50) |
| 4 year PFS (months) | 74.7 vs 34.9 | 46.2 vs 34.9 | 74.7 vs 46.2 |
| HR (95% CI) | 0.28 (0.12-0.67) | 0.72 (0.35-1.46) | 0.38 (0.14-1.05) |
| *P* value | **0.004** | 0.36 | 0.063 |
| **Loco-regional control (LRC)** | | | |
| Events (%) | 3 (12) vs 26 (53) | 6 (30) vs 26 (53) | 3 (12) vs 6 (30) |
| 4 year LRC (months) | 86.9 vs 43.1 | 62.3 vs 43.1 | 86.9 vs 62.3 |
| HR (95% CI) | 0.17 (0.05-0.55) | 0.49 (0.20-1.20) | 0.33 (0.08-1.32) |
| *P* value | **0.003** | 0.117 | 0.116 |
| **Overall survival (OS)** | | | |
| Events (%) | 6 (24) vs 28 (57) | 11 (55) vs 28 (57) | 6 (24) vs 11 (55) |
| 4 year OS (months) | 69.2 vs 40.9 | 34.9 vs 40.9 | 69.2 vs 34.9 |
| HR (95% CI) | 0.34 (0.14-0.81) | 0.94 (0.47-1.90) | 0.33 (0.12-0.89) |
| *P* value | **0.015** | 0.871 | **0.029** |

Univariate Cox regression analysis; HR= hazard ratio; CI= confidence interval; [a]HPV negative=matched HPV negative cases; [b]HPV positive= high risk HPV RNA-ISH positive; [c]p16 positive= high risk HPV RNA-ISH negative but showing moderate to strong diffused nuclear and cytoplasmic p16 staining in ≥70% tumor cells according to CAP (College of American Pathologist) criteria.

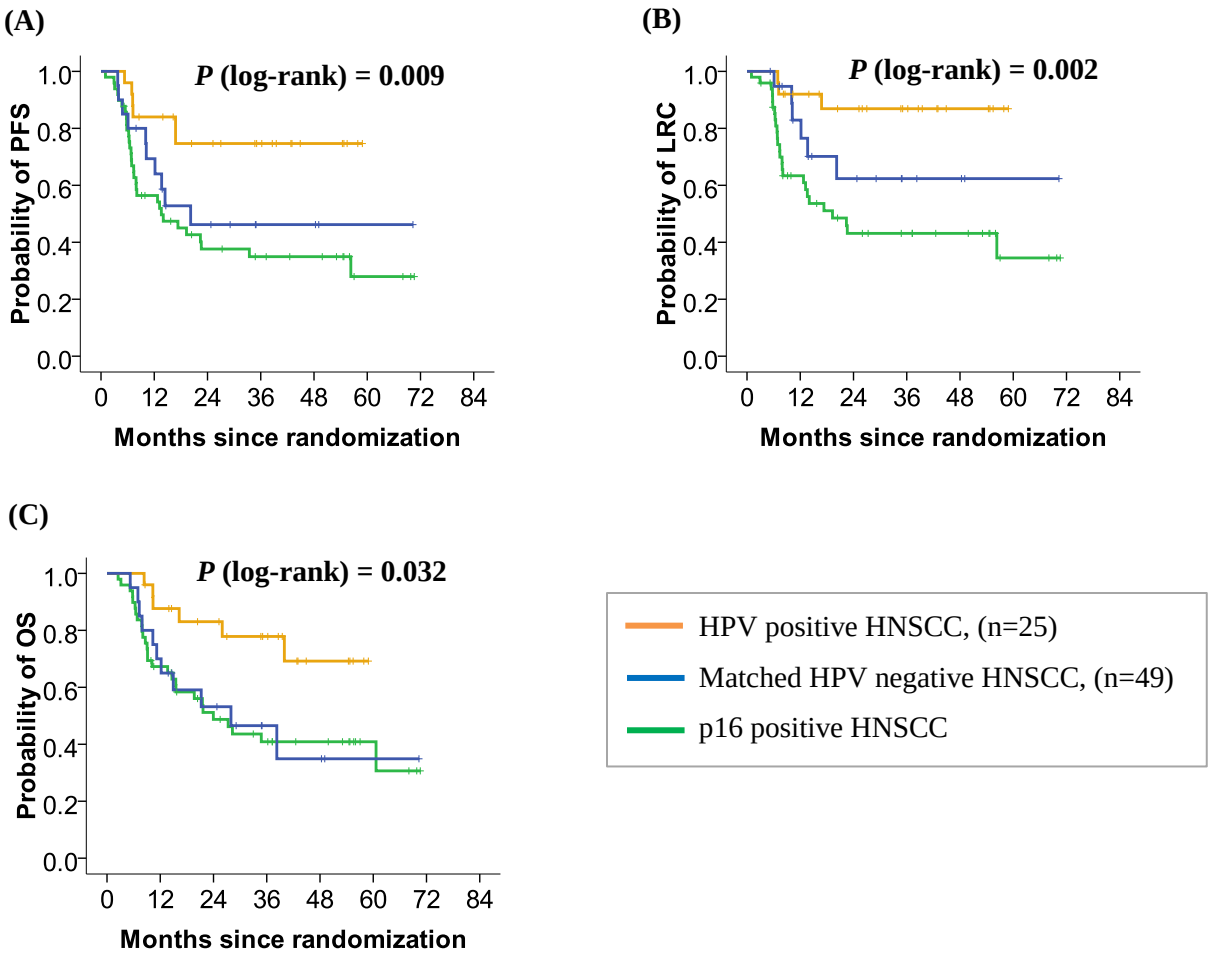


***Figure 40: Kaplan-Meier plots showing clinical outcomes in different study groups.*** *Progression frees survival (A), Loco-regional control (B) and Overall survival (C) in HPV positive, matched HPV negative and p16 positive groups.*

### 6.3. HPV status and p16 expression

High expression of nuclear p16 was observed in HPV positive ($P$<0.0001) and p16 positive ($P$<0.0001) groups as compared to the HPV negative group (Figure 41).

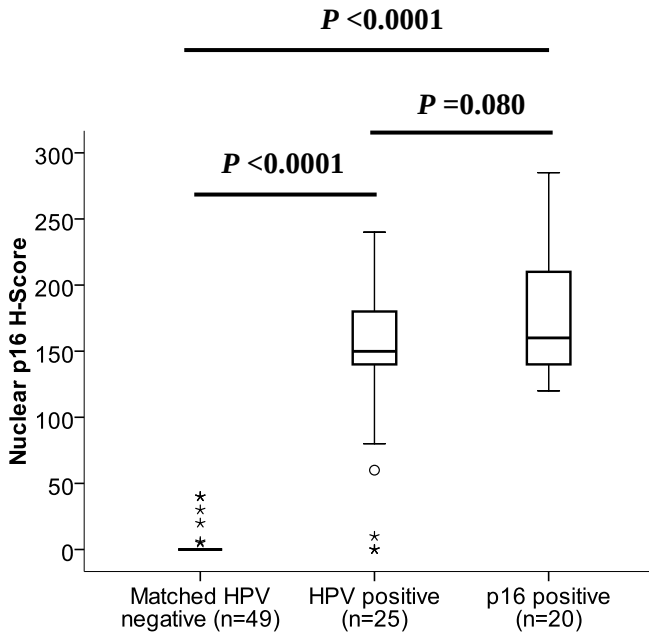


***Figure 41: Differential expression of p16 in three study groups***

*Box plots showing the difference in the expression of nuclear p16 in HPV positive, matched HPV negative and p16 positive*

## 6.4. Association of HPV status with expression of EGFR and pEGFR

The membrane expression of EGFR was observed in all subgroups. However, the expression levels of EGFR in HPV negative tumors were significantly higher than those in HPV positive tumors (*P*=0.018, Figure 42A).

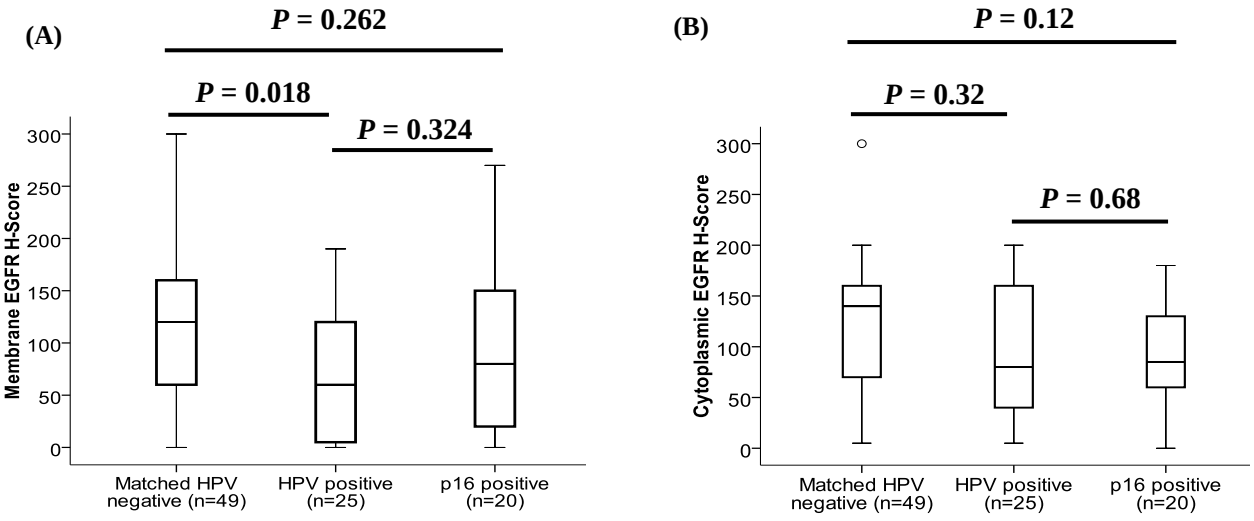


***Figure 42: Box plots showing the difference in the expression of EGFR***

*Membrane (A) and cytoplasmic EGFR (B) expression in HPV positive, matched HPV negative and p16 positive groups.*

The cytoplasmic expression levels of EGFR were not significantly different between the following subgroup pairs: HPV positive and HPV negative subgroups (p = 0.32); HPV positive and p16 positive subgroups (p = 0.68); and HPV negative and p16 positive subgroups (p = 0.12, Figure 42B). In contrast, the expression level of pEGFR dimers was downregulated in all subgroups. The expression of pEGFRY1068 was not detected in HPV positive tumors and only one HPV positive tumor showed expression of pEGFRY1173 (Figure 43 A-B).

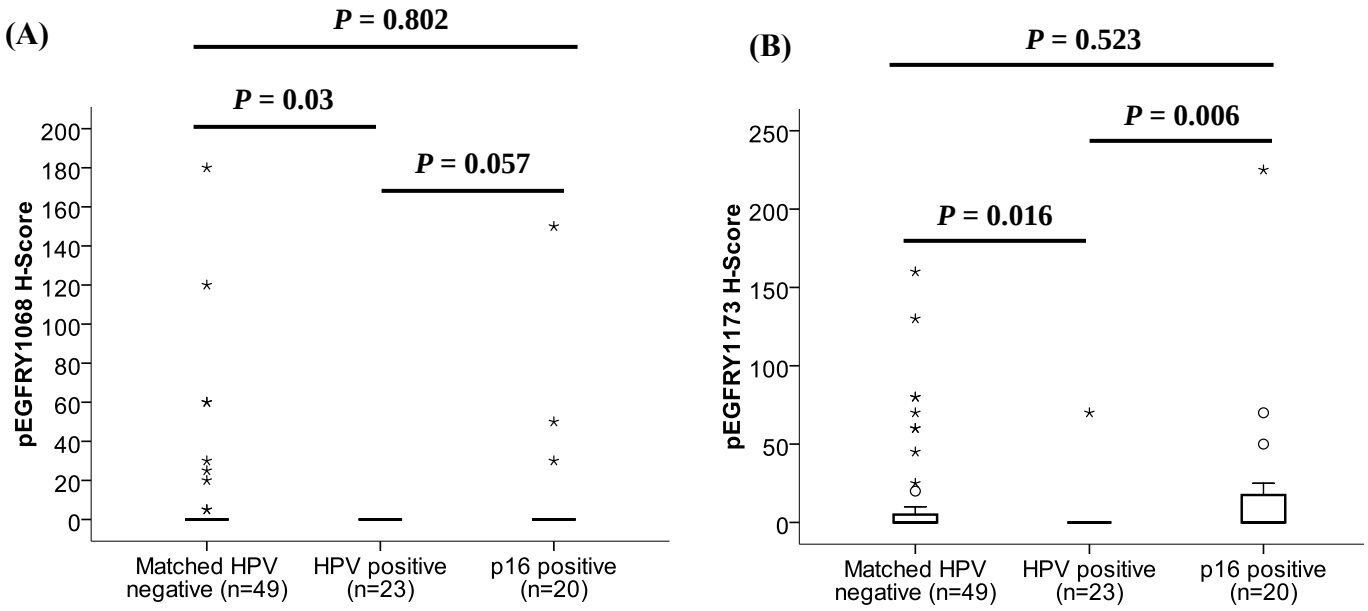


***Figure 43: Box plots showing the difference in membrane expression of pEGFR dimers.*** *pEGFRY1068 (A) and pEGFRY1173 (B) in HPV positive, matched HPV negative and p16 positive groups*

The expression levels of total EGFR (membranous or cytoplasmic) and pEGFR dimers were not markedly different between the HPV negative and p16 positive subgroups. Representative images of IHC staining of membrane EGFR and pEGFR dimers in HPV-positive and HPV negative tumors are shown in Figure 44.

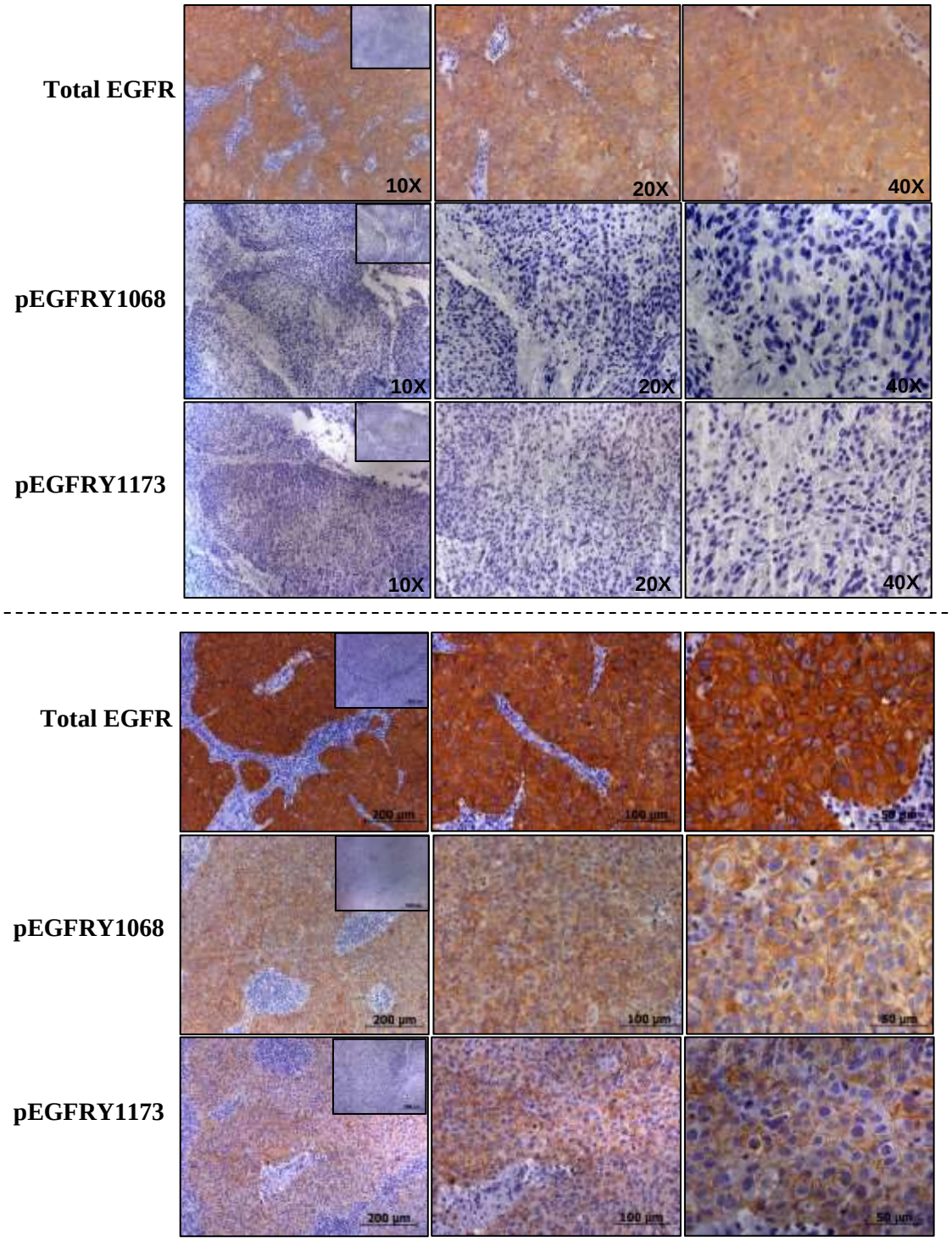


***Figure 44: Representative IHC staining of EGFR and pEGFR dimers***

*Upper panel shows expression in HPV positive HNSCC and lower panel shows expression in HPV negative HNSCC. Inset shows respective isotype control.*

## 6.5. Association of HPV status with expression of hypoxia markers

Representative images of IHC staining of HIF1α and CA9 in HPV positive and HPV negative tumors are shown in Figure 45.

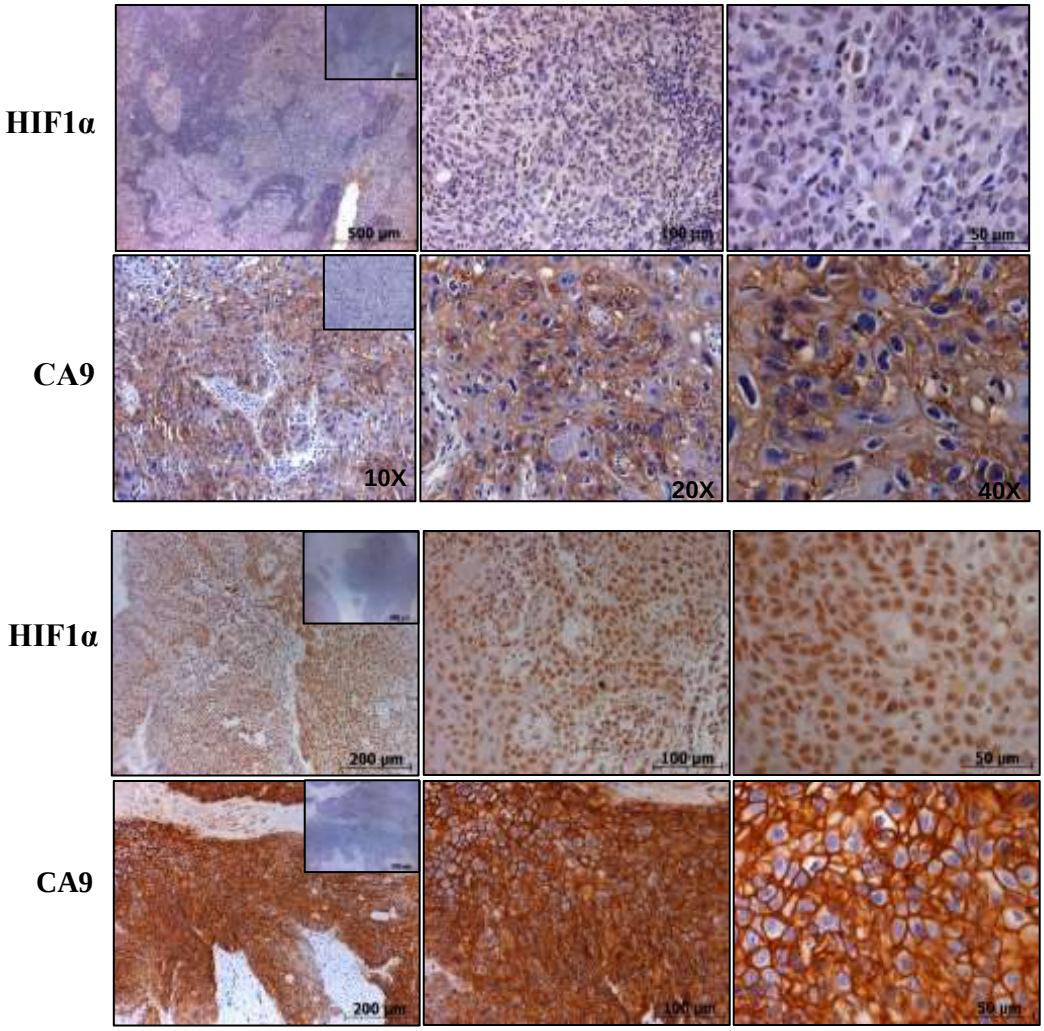


***Figure 45: Representative IHC staining of hypoxia markers***

*Upper panel shows expression of HIF1α and CA9 in HPV positive HNSCC and lower panel shows expression of HIF1α and CA9 in HPV negative HNSCC. Inset shows respective negative control.*

Compared with those in HPV positive HNSCCs, the expression levels of HIF1α were not significantly upregulated in HPV negative HNSCCs (*P*=0.067, Figure 46A). The expression level of HIF1α in the p16 positive subgroup was significantly higher than that in the HPV positive subgroup (*P*=0.013, Figure 46A). The expression level of HIF1α in the p16 positive subgroup was significantly higher than that in the HPV positive subgroup (*P*=0.205, Figure 46A). The expression of CA9 another hypoxia marker, did not differ between the groups (Figure 46B).

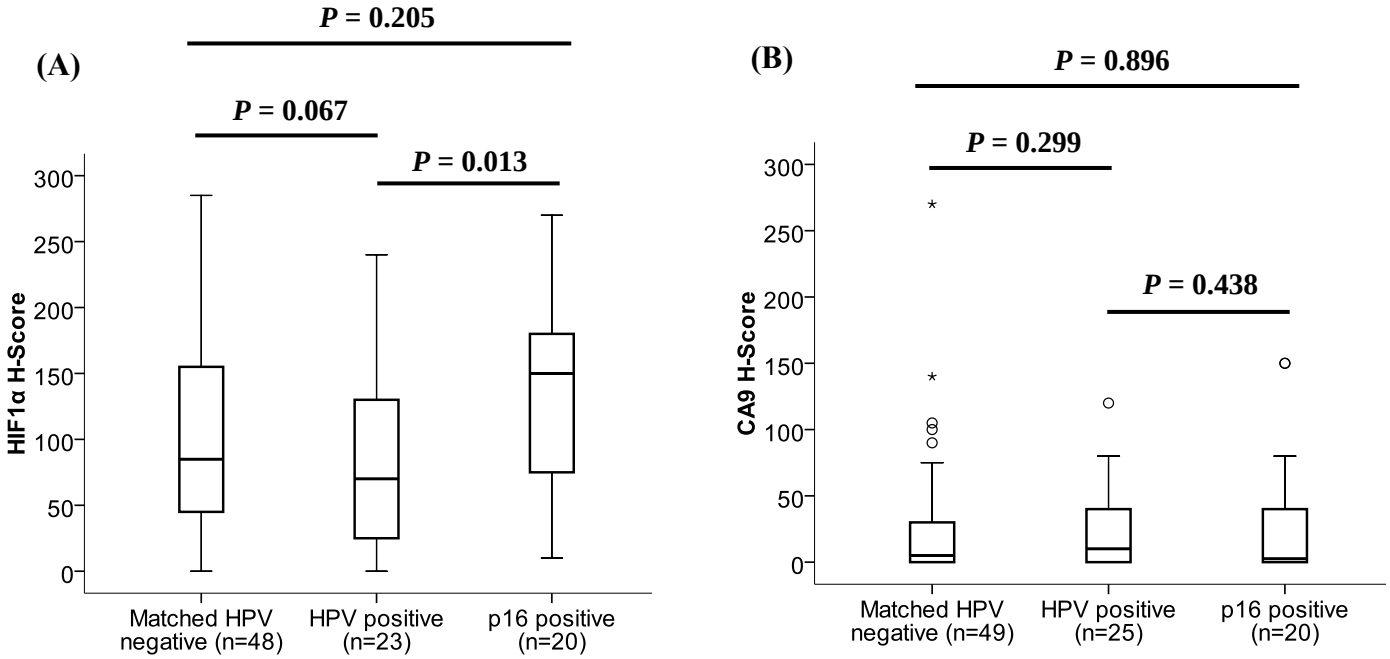


***Figure 46: Box plots showing the difference in expression of hypoxia markers***
*HIF1α (A) and CA9 (B) in HPV positive matched HPV negative and p16 positive groups.*

### 6.6. Association of HPV status with CD44 and CD44v6 expression

CD44 exhibited an incomplete membrane expression pattern in all three subgroups. HPV positive tumors showed low complete (*P* <0.0001) and incomplete (*P*=0.006) membrane CD44 expression than HPV negative tumors (Figure 47 A-B). In contrast, the expression level of

CD44v6, which exhibited a complete membrane expression pattern, in the HPV positive subgroup was significantly lower than that in the HPV negative (*P*=0.004) and p16 positive subgroups (*P* <0.0001, Figure 47C). The incomplete membrane expression of CD44v6 was not significantly different between the subgroups (Figure 47D).

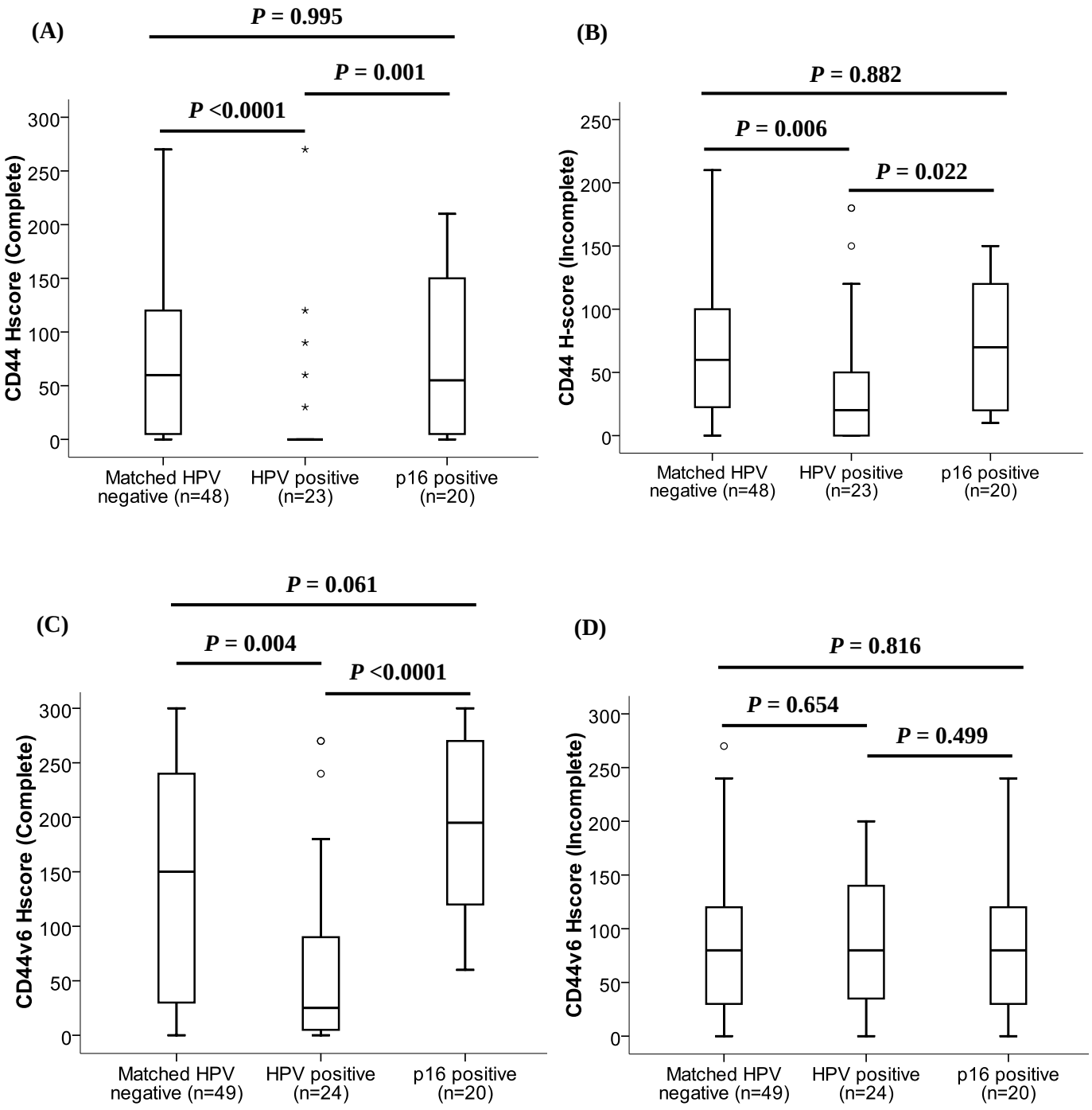

***Figure 47: Box plots showing the difference in membrane expression of CD44 and CD44v6***

*CD44 complete membrane (A), CD44 incomplete membrane (B), CD44v6 complete membrane (C) and CD44v6 incomplete membrane (D) expression in HPV positive, matched HPV negative and p16 positive groups.*

## 6.7. Association of HPV status with CD98hc, ALDH1A1 and SOX2 expression

The complete and incomplete membrane expression levels of CD98hc in HPV positive HNSCCs were significantly lower than those in HPV negative HNSCCs ($P$<0.0001 and $P$=0.019 respectively) or p16 positive HNSCCs ($P$=0.002 and $P$=0.031 respectively, Figure 48 A-B).

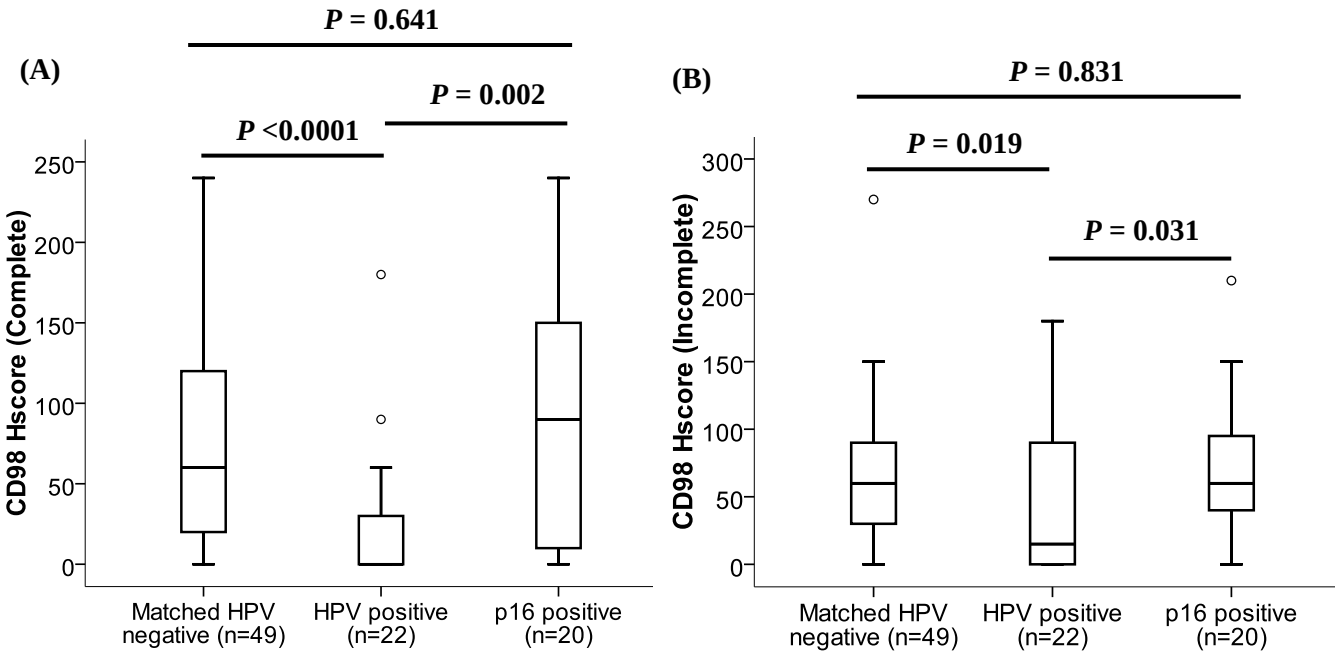


***Figure 48: Box plots showing the difference in membrane expression of CD98hc***

*CD98hc complete membrane (A) and CD98hc incomplete membrane (B) expression in HPV positive, matched HPV negative and p16 positive groups.*

The expression levels of ALDH1A1 or SOX2 were not significantly different between the subgroups **(Figure 49 A-B).** Interestingly, the expression levels of the potential CSC markers were not significantly different between the matched HPV negative and p16 positive subgroups. Representative images of IHC staining of putative CSC markers in HPV positive and HPV negative tumors are shown in **Figure 50 A-B.**

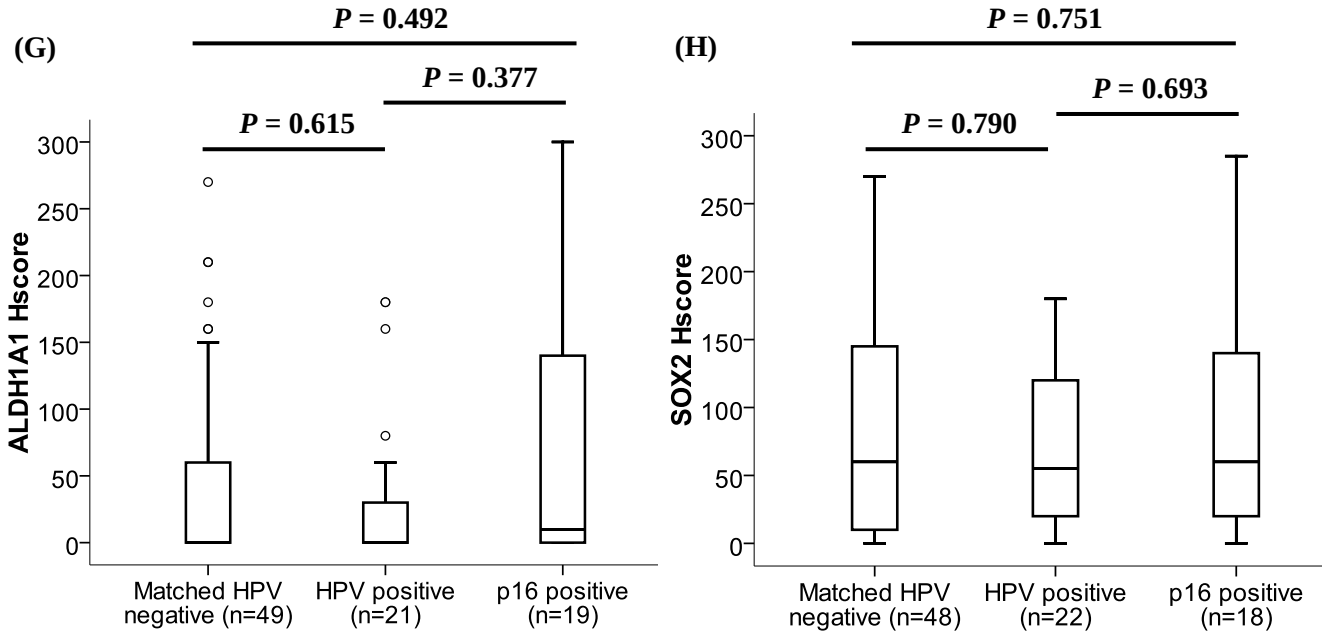


***Figure 49: Box plots showing the difference in expression of ALDH1A1 and SOX2***

*ALDH1A1 (A) and SOX2 (B) expression in HPV positive matched HPV negative and p16 positive groups.*

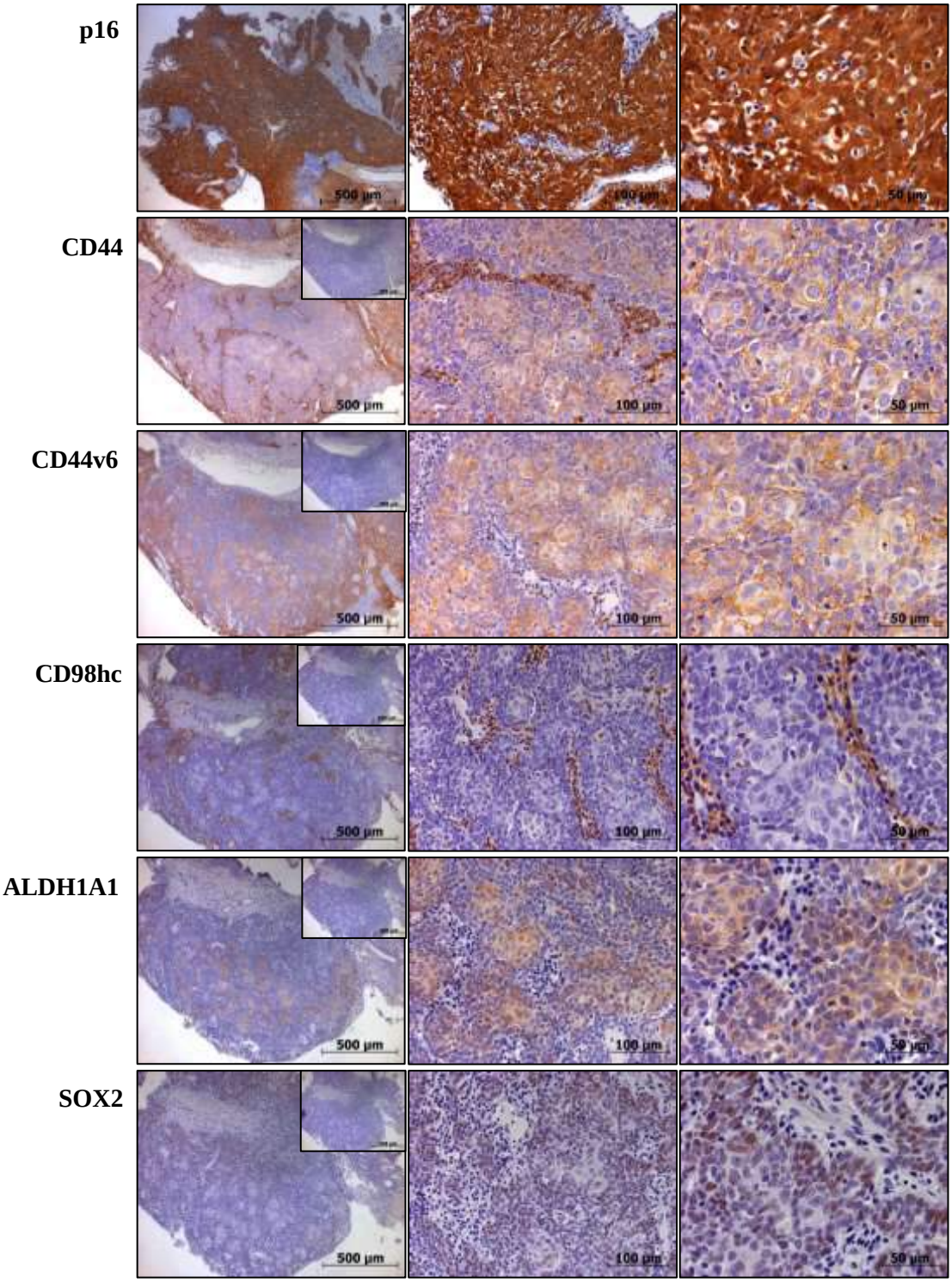


***Figure 50A: Representative IHC staining of p16 and different CSCs markers in HPV positive OPSCC (tonsil).*** *Inset shows respective negative control.*

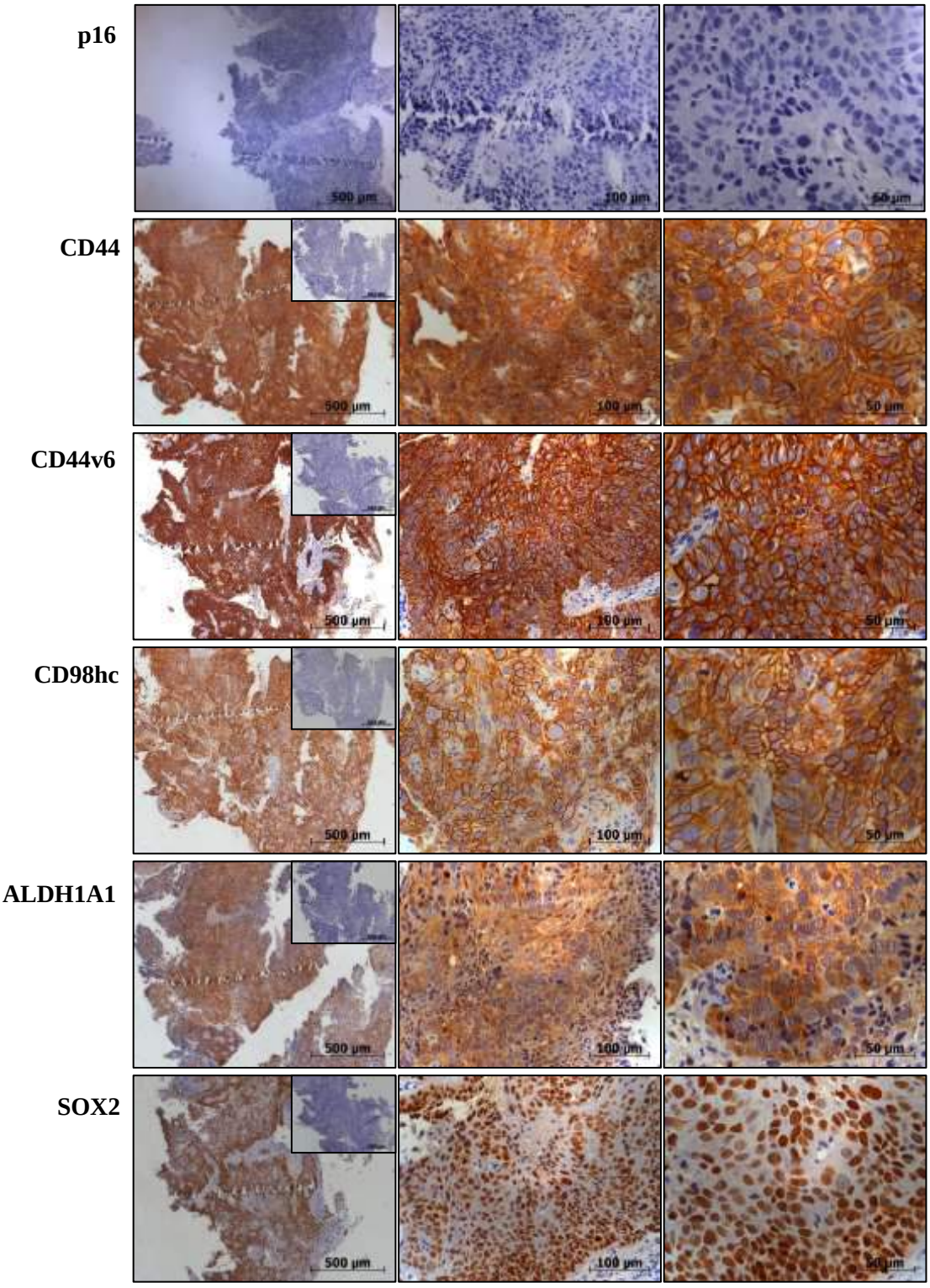


***Figure 50B: Representative IHC staining of p16 and different CSCs markers in HPV negative OPSCC (tonsil).*** *Inset shows respective negative control.*

## 6.8. Summary and Discussion

This study analyzed the clinical outcomes and the expression levels of different protein biomarkers in patients with HPV positive, HPV negative, or p16 positive/HPV negative HNSCC. The clinical and molecular profiles of HPV positive tumors were distinct from those of HPV negative tumors. However, the clinical and molecular profiles of p16-positive tumors lacking transcriptionally active HPV were similar to those of HPV negative HNSCCs.

In this study, HPV in HNSCCs was detected using RNA-ISH, which is a gold standard for confirming the presence of a transcriptionally active virus (47, 48). Although India has a high incidence of HNSCCs, various studies have suggested that the overall prevalence of HPV related HNSCCs is low in India (26). The prognostic value of HPV in OPSCC is established in the Western and European populations. However, there are limited studies on the prognostic value of HPV in the Indian population (121). In this study, HPV positive status was associated with a significantly improved prognosis in patients with OPSCC, which was consistent with the findings in the global population (122). p16 overexpression is a surrogate marker for HPV infection. However, HNSCC tumors seldom show HPV-independent p16 overexpression, which is not associated with mutation or amplification of *CDKN2A* (123, 124). In this study, p16 expression was observed in 4.7% of HNSCC tumors that yielded negative results for HPV in RNA-ISH analysis. The clinical outcomes were not significantly different between the p16 positive and HPV negative HNSCCs. Compared with the HPV positive subgroup, the p16 positive subgroup exhibited a significantly worse OS and a non-significantly worse PFS and LRC. Similar results have been reported by different groups (123, 124).

HPV positive tumors exhibited lower expression levels of EGFR and pEGFR dimers. Inverse correlation between HPV status and EGFR expression is well established in the literature (125). However, limited studies have analyzed the correlation between pEGFR expression and HPV status. The expression levels of HIF1α in HPV positive tumors were not significantly lower than those in HPV negative tumors. However, the expression of CA9 was not associated with HPV status. Previous studies have not reported the correlation between HPV and hypoxia or HIF1α expression in patients with HNSCC (126-128). This study demonstrated that the expression levels of HIF1α and CA9 were not significantly different between the HPV negative and p16 positive subgroups.

There are conflicting reports on the correlation between CSC population and HPV status, which may be attributed to the analysis of different CSC markers in different studies (55-60). Limited studies have comprehensively analyzed different CSC markers in clinical samples. This study comprehensively evaluated the expression of five putative CSC markers based on HPV status. Interestingly, the expression levels of CD44, CD44v6, and CD98hc in HPV positive tumors were significantly lower than those in HPV negative and p16 positive tumors. However, the expression levels of ALDH1A1 or SOX2 were not correlated with HPV status. Previous studies have reported that lower expression of CD44 and CD98hc is associated with HPV status in OPSCC; additionally, the high expression of CD98hc is a negative prognostic marker in HPV positive OPSCC (57, 59). As the CSC population is heterogeneous, the analysis of the expression of a single CSC marker cannot distinguish CSCs from non-CSCs. This indicated that the phenotype of CSC is more important than the number of CSCs. Among the five CSC markers, the expression levels of CD44, CD44v6, and CD98hc were negatively correlated with HPV status. However, the expression levels of ALDH1A1 and SOX2 were not correlated with HPV

status. The expression levels of all CSC markers were similar between the HPV negative and p16 positive subgroups.

This study is associated with few limitations. The study cohort comprised a small number of patients with HPV positive tumors. Additionally, the expression levels of biomarkers were determined using IHC analysis, which is a semi-quantitative method.

In summary, this study demonstrated that India has a low prevalence of transcriptionally active HPV, which was strongly associated with improved clinical outcomes in patients with OPSCC. To the best of our knowledge, this is the first study to comprehensively analyze the expression of different protein biomarkers in HPV positive, HPV negative, and p16 positive/HPV negative HNSCC tumor tissues. The clinical and molecular profiles of patients with p16 positive/ HPV negative HNSCC were similar to those of patients with HPV negative HNSCC but significantly different from those of patients with HPV positive HNSCC; their demarcation is required when considering the de-escalation treatment strategies. Therefore, RNA-based HPV detection methods are essential for determining the clinical and biological relevance of HPV status.

# 7.

# Overall

# Summary and Conclusions

## 7.1. Summary of the work

In the present study, we have evaluated both prognostic and predictive significance of HIF1α, EGFR based biomarkers, CD44, CD44v6, CD98, ALDH1A1, and SOX2 in HPV negative LA-HNSCC patients treated either with CRT or NCRT in a phase 3-randomized study. We also analyzed differences in clinical and molecular profiles of HPV positive, matched HPV negative, and p16 positive (HPV negative) HNSCC patients.

## 7.2. Prognostic and predictive significance of HIF1α and EGFR based biomarkers in HPV negative LA-HNSCCs

Our results suggest that nuclear HIF1α expression is an independent negative prognostic factor in HPV negative HNSCC patients. The addition of nimotuzumab to CRT significantly improves the clinical outcomes in high HIF1α expressing patients. HIF1α status showed significant qualitative interaction with treatment effect. EGFR or pEGFR expression or EGFR gene copy number did not have any prognostic or predictive significance in these patients. Ours is the first study showing both prognostic and predictive significance of nuclear HIF1α expression and it needs to be validated independently.

## 7.3. Prognostic and predictive significance of different putative cancer stem cell markers in HPV negative LA-HNSCCs

CD98hc expression was independently associated with poor clinical outcomes and thus is a negative prognostic factor in HPV negative LA-HNSCCs patients. CD98hc did not show any predictive association. CD44 and CD44v6 did not show any prognostic role, however; low expression of CD44 or CD44v6 can predict clinical benefit from the addition of nimotuzumab to

cisplatin-radiation. CD44 and CD44v6 status showed significant qualitative interaction with treatment effect for OS. Ours is the first study showing the predictive impact of complete membrane expression of CD44 and CD44v6 for NCRT treatment response. Expression of ALDH1A1 and SOX2 did not show any significant prognostic or predictive significance.

### 7.4. Correlation of transcriptionally active human papillomavirus status with the clinical and molecular profiles of HNSCC

Our study showed that India has a low prevalence of transcriptionally active HPV in HNSCCs which was strongly associated with better clinical outcomes and significantly low expression of EGFR, CD44, CD44v6, and CD98hc. Patients with tumors showing p16 positivity but lacking active HPV have similar clinical and molecular profiles as that of HPV negative HNSCC but have significantly distinct profiles compared to HPV positive HNSCC. Ours is the first study comprehensively analyzing different protein biomarker expression in HPV positive, matched HPV negative, and p16 positive (HPV negative) HNSCC tumors tissues.

# 8.

# Concluding remarks and

# Future directions

Advances in the understanding of tumor progression at molecular levels have opened doors for targeted cancer therapeutics. Prognostic biomarkers are extensively studied in HNSCCs, but they have limited utility in patients' treatment decisions. While the identification of predictive biomarkers is a pressing need to enable the selection of patients for a specific treatment. However, there are very limited predictive biomarker studies in HNSCC for targeted therapies. Cetuximab induced skin rashes has been reported as potential predictive biomarker for cetuximab treatment response, however is has not been established as a predictive biomarker. In addition, skin rashes highly affect the quality of life of the patients Pinto et al., Oncologist, 2011; Petrelli et al., Target Oncol. 2013). Compared to prognostic studies, predictive biomarker studies are more challenging as to identify an interaction between any biomarker and treatment effect a larger sample size is needed, and also a randomized setting is considered to be ideal where experimental treatment is compared with conventional treatment. In randomized clinical studies, patients are treated uniformly in treatment groups and randomization allows an unbiased comparison of the treatment effects as important confounding factors as well as the distribution of biomarkers is balanced between the treatment groups. Therefore, we have evaluated the prognostic and predictive role of different biomarkers in a large cohort of HPV-negative LA-HNSCC patients treated in a randomized setting.

In literature, there is no consensus regarding the method of evaluation of IHC staining for studied biomarkers (percentage of tumor cell stained or intensity of staining or both). In the current study, IHC staining was evaluated independently by the two pathologists by deriving HScore, and cases showing discrepancy were jointly resolved to reduce the bias. For biomarker expression analysis with continuous data, selection of the appropriate cutpoint for dichotomizing patients into expression subgroups remains a difficult decision as there is no consensus among

studies. The median value is often used as the cutpoint, but it may not be optimal for all biomarkers, and using a prespecified cutpoint will increase the probability of failure in detecting important associations. Therefore, we dichotomized the biomarker expression at different possible cutpoints and carried out prognostic and predictive analysis at each cutpoint for all the protein biomarkers.

Our study has shown that HIF1α, CD44, and CD44v6 are potential candidate predictive biomarkers in HPV negative HNSCC patients for NCRT treatment response. We also showed that HIF1α is a strong independent negative prognostic factor in these patients. Additionally, our study also indicated a low prevalence of active HPV infection in Indian HNSCC patients. RNA based methods for HPV detection is essential for determining the clinically and biologically relevant HPV status.

In the future, other frequently altered downstream molecules of EGFR signaling including the PI3K-AKT-mTOR pathway and angiogenic markers need to be evaluated in combination for their predictive potential in HNSCCs (129, 130). In addition to nimotuzumab, anti-angiogenic drugs can be explored for high HIF1α expressing patients (131). Clinical trials should also include parallel biomarker studies to effectively identify and validate predictive biomarkers.

# Appendix

**TATA MEMORIAL CENTRE**

**(TATA MEMORIAL HOSPITAL)**

**Informed Consent form for Patients (Sample)**

**Title of the project:** Analysis of genetic host factors, HPV, EGFR and Hypoxia markers and their association with outcome in subjects with locally advanced Squamous cell carcinoma of head and neck (LASCCHN)."

**Principal Investigators :** Dr Manoj Mahimkar, Dr Kumar Prabhash

**Co- Principal Investigators :** Dr Anil D'Cruz, Dr Sarbani Laskar, Dr Shubhada Kane, Dr Shashikant Juvekar.

Thank you for reading this consent brochure. Please take your time to decide if you want to participate in this study. Discuss this with your family and friends if you would find this helpful. Please feel free to ask any questions that will help you make your decision.

**What is this project about?**

The proposed study aims to understand the interrelationship between genetic & molecular changes occurring in head and neck cancers and their association with clinical outcome. As a part of treatment cancer patients receive chemo and radiation therapy in combination with surgery. In spite of complete treatment in many cases there is recurrence of disease which may lead to shorter survival. Work carried out globally has indicated that genetic and molecular biomarkers are useful in more accurate predication of disease recurrence and survival. However majority of the studies have used single biomarkers markers and comprehensive analysis with multiple markers is lacking. Hence in this study we are planning to use battery of these biomarkers which may benefit the patients in future.

**How was I selected for the study?**

Your treating doctor/s at TMC are either suspecting or have confirmed a diagnosis of cancer /tumor for which you have been advised Biopsy/Surgery at TMC. You were specifically chosen for this study because your medical team feels that:

a. After your routine biopsy / surgery as is routinely done for such cases at TMC, excess tumor tissue may be left after completing the standard pathology tests required for such cases.

b. You may be willing to donate this excess tissue & additional blood/ body fluids for genetic/genomics & other research.

c. You are likely to complete the standard treatment advised to patients with this type & stage of cancer / tumor.

d. You are expected to come back regularly to TMC for routine follow up check ups

Your participation is voluntary. Your treatment will not be affected by your decision to participate / not to participate in the study

**How many patients will be recruited?**

We are planning to recruit 536 patients with advanced stage head and neck cancers.

**Your participation in this project means agreeing to the following actions:**

a. Use of previously and newly collected tissues: Your consent will allow us to use, for research purposes, any tissue samples that may have been taken from you previously, or will be taken before or during any planned surgery / biopsy or procedure. In addition to the present study these samples will also be used for additional genetic and molecular studies which will be carried out in future after obtaining approval from IRB.

b. Consent for additional blood / saliva sample: By giving your consent you agree to donate and allow us to collect and use your blood (approximately 15 ml or 2 teaspoons), saliva for analysis of genetic and other molecular markers. DNA or other biological or chemical molecules will be extracted from these samples. It is possible that your sample will undergo genetic screening in which we determine many or all the features of your DNA that distinguish it from other people's DNA.

c. This information about genetic and other molecular studies will not be returned to you. However, there is a small chance (less than 5%) that genetic / molecular studies on blood or normal tissue may identify a germline deleterious mutation/s. In such case your treating doctor at TMC may contact you for genetic counseling and repeat genetic testing for this mutation if they feel that it could help your medical management, prevent 2nd cancers or facilitate prevention or early detection of cancers in your family.

d. Access to Health Information: By giving your consent you give us permission to access identified health information kept about you that is relevant to medical research. Such medical records may originate from hospitals general practice records, diagnoses by private specialists you have seen in the past, and information that is held on you by cancer registries, registrars of birth and death, administrative health databases etc. Images of tumor histology and radiology may also be stored and shared with investigators if necessary as per TMC controlled access policy.

**Option for repeat blood and or saliva sample in the future.**

Do you give your permission for the Project team to collect your blood / saliva sample during your routine follow up visits or when a tumor recurrence is suspected or confirmed. You can still participate in the project even if you decline

Yes:_____No:_____

**Option for re-contact in the Future:**

Do you give your permission for the project team to re-contact you in the future if necessary and request you to provide additional samples or information related to the project or to invite you to participate in a new study. You can still participate in the project even if you decline to be re-contacted

Yes_____No _____

**How will my samples and data be stored and protected?**

Your tissue and other samples, the data derived from any analyses of those samples and your personal information found in your health records will be coded to protect your confidentiality. Staff at TMC will remove personal identifiers such as your name and address, and replace them with a unique code. This unique code will enable us to link the information from different datasets, for example, your health record to your samples. Only the designated project staff will hold the key that connects your code to data that can identify you.

Your coded samples may also be shared for approved studies between several teams in the country or outside after obtaining necessary ethical and regulatory approvals. Your coded samples may be shared with other researchers in India or outside for quality control purposes. Tata Memorial Centre will be the custodian of your samples.

**Who can access my data?**

The data will be organized and placed into two databases - Open and Controlled-Access.

Open-access: information in this database will be publicly accessible, but will not contain any information that could be used to identify you specifically.

Controlled-access: This database will contain only your coded medical information and information from the more detailed analyses of your coded samples. This will be accessible only to investigators in the team.

Your identity will be kept confidential in all presentations, reports or publications. All data will be presented as group data, rather than individual data.

**What are the benefits of taking part in this study?**

It is not expected that you will receive any direct individual / health benefits from the work done in this project and will be realized many years from now, and may largely help future generations. The knowledge gained from the study may be useful in designing better treatment strategies to improve the quality of patient's life in future. General research results will be published in peer reviewed journals.

**Are there any risks for me?**

a. Physical Risks: There are very minor risks involved in your participation beyond inconvenience and discomfort. Some people experience bruising or may faint after giving blood.

However all procedures will be carried out by suitably qualified staff as your welfare is our priority. The storage of your biological samples involves minimal risk.

b. Privacy and Security Risks: There is a remote risk that the genetic information generated by the project could eventually be linked to genetic or medical information in other databases. However, we will make every effort to protect confidentiality of your information.

c. Access to this information will not be offered to third parties such as employers, insurance companies or other family members unless it is requested by you, required by law or a court order.

**What if something is found?**

Your samples and data are not intended to be used for your diagnosis or treatment and therefore no individual results will be returned to you. However, there is a small chance (less than 5%) that genetic studies on blood or normal tissue may identify a germline deleterious mutation (harmful change in a gene known to cause hereditary cancers) in a high penetrance gene. In such case your treating doctor may contact you for genetic counseling and repeat genetic testing for this mutation if they feel that it could help your medical management, prevent 2nd cancers or facilitate prevention or early detection of cancers in your family.

**Will there be any commercialization?**

The use of your data and samples might one day lead to the commercialization of a medical or genetic test or product. A university / hospital, researchers, a commercial company or both working in partnership may do this and may benefit financially. You will not derive any personal financial advantage from this commercialization.

**Whom to contact if I have concerns?**

If you decide to participate but have any concerns in the future, you can telephone us at Dr. Manoj Mahimkar (022-27405049), Dr Kumar Prabhash (022-24177214). Alternatively, you can write a letter to the project coordinator, or to Dr. Vikram Gota, (022-27405130), Member Secretary, TMC-ACTREC Institutional Review Board, ACTREC, Tata Memorial Centre, Kharghar, Navi Mumbai 410 210 or email me mmahimkar@actrec.gov.in.

**Will I be paid for participation (Costs/Compensation/ Reimbursement)?**

Your participation is on a voluntary basis. No charge would be taken from you for participation or you will not be paid for any tests or treatment. Taking part in this study will not lead to added costs to your insurance company.

**How can I withdraw from the Project?**

You are free to withdraw at any time from your participation in the project and without giving any reason. If you withdraw from the study prior to its completion, you will receive the usual standard of care for your disease, and your non-participation will not have any adverse effects on subsequent medical treatment or relationship with the treating physician. You can withdraw by telephoning us or by writing to the Project Coordinator, ACTREC. You will receive a letter to confirm your withdrawal. If you withdraw from the study, your remaining samples will be

destroyed and but the coded data derived from any analysis already done on your samples may be used without revealing any personal.

Kindly note that it is investigators discretion to terminate the project or your participation in the project based on the newer information emerging from time to time. By participating in the study, the patient also understand that you need to follow up regularly with your treating physicians.

**Consent to Participate in Study**

I have read the above information and agree to participate in this study. By signing this / providing my thumb impression, I agree to the use of my previously and/or newly collected tissue, the collection of blood, saliva or DNA and to allow access to my health information. I also agree that these samples may be used in future for additional genetic and molecular studies which will be planned in future. I have also made my choice regarding additional blood / saliva samples during follow-up or at recurrence; re-contact in the future. I have received a copy of this form.

Participant's name (print) _____

Participant's signature _____

Address (in capital letters)_____

_____

Tel No._____ Date _____

Witness's name (print)_____

Witness's signature_____

Tel No._____ Date _____

PI or the person administering the consent: Name (Print) & signature

| Table 1: Demographic details of the HPV positive (RNA-ISH positive) HNSCC patients | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sr No.** | **Gender** | **Age** | **Major tumor site** | **Tumor subsite** | **Tobacco chewing*** | **Bidi smoking#** | **Cigarette smoking#** | **Alcohol** | **p16 IHC status** | **HPV DNA-PCR** |
| 1 | Female | 61 | Oropharynx | Base of tongue | Current, light | Never | Never | Never | Positive | Positive |
| 2 | Male | 53 | Oropharynx | Base of tongue | Never | Never | Never | Never | Positive | Negative |
| 3 | Female | 40 | Oropharynx | Base of tongue | Former, light | Never | Never | Never | Negative | Negative |
| 4 | Female | 50 | Oropharynx | Base of tongue | Current, heavy | Never | Never | Never | Positive | Positive |
| 5 | Male | 44 | Oropharynx | Base of tongue | Never | Current, <10 pack-year | Never | Never | Positive | Negative |
| 6 | Male | 35 | Oropharynx | Tonsil | Current, heavy | Current, <10 pack-year | Current, <10 pack-year | Never | Positive | Positive |
| 7 | Male | 49 | Oropharynx | Tonsil | Never | Never | Never | Never | Positive | Positive |
| 8 | Female | 49 | Oropharynx | Tonsil | Never | Never | Never | Never | Positive | Positive |
| 9 | Male | 20 | Oropharynx | Tonsil | Never | Never | Never | Never | Positive | Negative |
| 10 | Male | 42 | Oropharynx | Tonsil | Never | Current, <10 pack-year | Never | Never | Positive | Negative |
| 11 | Male | 36 | Oropharynx | Tonsil | Never | Never | Never | Former | Negative | Negative |
| 12 | Female | 42 | Oropharynx | Tonsil | Never | Never | Never | Never | Negative | Negative |
| 13 | Male | 34 | Oropharynx | Tonsil | Current, heavy | Current, <10 pack-year | Never | Never | Positive | Positive |
| 14 | Female | 46 | Oropharynx | Tonsil | Never | Never | Never | Never | Positive | Positive |
| 15 | Male | 66 | Oropharynx | Tonsil | Never | Never | Former, <10 pack-year | Never | Positive | Positive |
| 16 | Male | 54 | Oropharynx | Tonsil | Current, light | Current, <10 pack-year | Current, <10 pack-year | Current | Positive | Positive |
| 17 | Male | 36 | Oropharynx | Tonsil | Never | Never | Never | Current | Positive | Positive |
| 18 | Female | 40 | Oropharynx | Tonsil | Current, light | Never | Never | Never | Negative | Positive |
| 19 | Female | 45 | Larynx | Glottis | No info | No info | No info | No info | Positive | NA |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 20 | Male | 20 | Larynx | Glottis | Never | Never | Never | Never | Positive | Positive |
| 21 | Female | 31 | Larynx | Supraglottis | Never | Never | Never | Never | Negative | Positive |
| 22 | Female | 26 | Larynx | Supraglottis | Never | Never | Never | Never | Positive | Positive |
| 23 | Female | 42 | Larynx | Supraglottis | Never | Never | Never | Never | Positive | Negative |
| 24 | Male | 54 | Hypopharynx | Pyriform sinus | Never | Never | Never | Never | Positive | Positive |
| 25 | Male | 65 | Hypopharynx | Pyriform sinus | Never | Never | Former, <10 pack-year | Never | Negative | Positive |

*Tobacco chewing is light (if chewing index is <80) or heavy (if chewing index is ≥80); Tobacco chewing index is calculated as frequency of chewing event per day multiplied by duration in years; #A pack-year is defined as the equivalent of smoking one pack of bidis (25 bidis/pack) or one pack of cigarettes (10 cigarettes/pack) per day for 1 year.

**Table 2 : Demographic details of the matched HPV negative HNSCC patients**

| Sr No. | Sex | Age | Major tumor site | Tumor subsite | Tobacco chewing | Bidi smoking | Cigarette smoking | Alcohol use | p16 IHC status | HPV DNA-PCR |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Male | 56 | Oropharynx | Base of tongue | Never | Current, >10 pack-year | Never | Former | Negative | NA |
| 2 | Male | 45 | Oropharynx | Base of tongue | Former, light | Never | Never | Never | Negative | Negative |
| 3 | Female | 51 | Oropharynx | Base of tongue | Never | Never | Never | Never | Negative | Negative |
| 4 | Male | 51 | Oropharynx | Base of tongue | Current, heavy | Never | Never | Current | Negative | Negative |
| 5 | Male | 55 | Oropharynx | Base of tongue | Never | Current, <10 pack-year | Never | Never | Negative | Negative |
| 6 | Male | 45 | Oropharynx | Base of tongue | Current, heavy | Never | Current, <10 pack-year | Current | Negative | Negative |
| 7 | Female | 50 | Oropharynx | Base of tongue | Current, heavy | Current, <10 pack-year | Never | Never | Negative | Negative |
| 8 | Male | 57 | Oropharynx | Base of tongue | Never | Current, >10 pack-year | Current, <10 pack-year | Current | Negative | Negative |
| 9 | Male | 47 | Oropharynx | Base of tongue | Never | Current, <10 pack-year | Current, <10 pack-year | Current | Negative | Negative |
| 10 | Male | 54 | Oropharynx | Base of tongue | Never | Current, >10 pack-year | Never | Former | Negative | Negative |
| 11 | Male | 41 | Oropharynx | Base of tongue | Current, light | Current, <10 pack-year | Never | Never | Negative | Negative |
| 12 | Male | 51 | Oropharynx | Base of tongue | Current, heavy | Never | Current, >10 pack-year | Current | Negative | Negative |
| 13 | Male | 59 | Oropharynx | Base of tongue | Current, heavy | Never | Never | Never | Negative | Negative |
| 14 | Male | 57 | Oropharynx | Base of tongue | Current, light | Current, <10 pack-year | Never | Current | Negative | Negative |
| 15 | Male | 57 | Oropharynx | Base of tongue | Former, heavy | Never | Never | Never | Negative | Negative |
| 16 | Male | 35 | Oropharynx | Base of tongue | Current, light | Current, <10 pack-year | Never | Current | Negative | Negative |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 17 | Male | 57 | Oropharynx | Base of tongue | Never | Former, >10 pack-year | Never | Former | Negative | Negative |
| 18 | Female | 55 | Oropharynx | Base of tongue | Never | Current, >10 pack-year | Never | Never | Negative | NA |
| 19 | Male | 59 | Oropharynx | Base of tongue | Current, light | Current, <10 pack-year | Never | Never | Negative | Negative |
| 20 | Male | 60 | Oropharynx | Post pharyngeal wall | Former, light | Current, <10 pack-year | Never | Current | Negative | Negative |
| 21 | Female | 44 | Oropharynx | Post pharyngeal wall | Former, light | Never | Never | Never | Negative | Negative |
| 22 | Male | 67 | Oropharynx | Post pharyngeal wall | Never | Never | Current, >10 pack-year | Never | Negative | Negative |
| 23 | Female | 40 | Oropharynx | Soft palate | Current, heavy | Never | Never | Never | Negative | Negative |
| 24 | Female | 45 | Oropharynx | Soft palate | Current, heavy | Never | Never | Never | Negative | Negative |
| 25 | Male | 65 | Oropharynx | Tonsil | Never | Current, <10 pack-year | Never | Current | Negative | Negative |
| 26 | Male | 45 | Oropharynx | Tonsil | Never | Current, <10 pack-year | Never | Current | Negative | Negative |
| 27 | Male | 31 | Oropharynx | Tonsil | Never | Never | Never | Never | Negative | Negative |
| 28 | Female | 47 | Oropharynx | Tonsil | Never | Current, <10 pack-year | Never | Never | Negative | Negative |
| 29 | Male | 61 | Oropharynx | Tonsil | Former, light | Former, <10 pack-year | Never | Never | Negative | Negative |
| 30 | Female | 42 | Oropharynx | Tonsil | Current, light | Never | Never | Never | Negative | Negative |
| 31 | Female | 45 | Oropharynx | Tonsil | Never | Never | Never | Never | Negative | Negative |
| 32 | Male | 54 | Oropharynx | Tonsil | Never | Current, >10 pack-year | Current, <10 pack-year | Former | Negative | Negative |
| 33 | Male | 59 | Oropharynx | Tonsil | Former, heavy | Never | Former, <10 pack-year | Former | Negative | Negative |
| 34 | Male | 57 | Oropharynx | Tonsil | Never | Current, >10 pack-year | Never | Former | Negative | Negative |
| 35 | Male | 47 | Oropharynx | Tonsil | Current, light | Former, | Never | Former | Negative | Negative |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | <10 pack-year | | | | |
| 36 | Male | 48 | Oropharynx | Tonsil | Never | Current, >10 pack-year | Never | Current | Negative | Negative |
| 37 | Female | 65 | Larynx | Supraglottis | Current, heavy | Never | Never | Never | Negative | Negative |
| 38 | Female | 50 | Larynx | Supraglottis | Never | Never | Never | Never | Negative | Negative |
| 39 | Male | 54 | Larynx | Supraglottis | Never | Current, <10 pack-year | Never | Current | Negative | Negative |
| 40 | Female | 35 | Larynx | Supraglottis | Never | Never | Never | Never | Negative | Negative |
| 41 | Female | 55 | Larynx | Supraglottis | Never | Never | Never | Never | Negative | Negative |
| 42 | Female | 28 | Larynx | Supraglottis | Never | Never | Never | Never | Negative | Negative |
| 43 | Female | 48 | Hypopharynx | Post cricoid | Never | Never | Never | Never | Negative | Negative |
| 44 | Female | 32 | Hypopharynx | Post cricoid | Never | Never | Never | Never | Negative | Negative |
| 45 | Male | 37 | Hypopharynx | Post cricoid | Never | Never | Never | Never | Negative | Negative |
| 46 | Male | 58 | Hypopharynx | Pyriform sinus | Current, heavy | Current, >10 pack-year | Never | Never | Negative | Negative |
| 47 | Male | 41 | Hypopharynx | Pyriform sinus | Never | Never | Never | Never | Negative | Negative |
| 48 | Female | 59 | Hypopharynx | Pyriform sinus | Never | Never | Never | Never | Negative | Negative |
| 49 | Male | 50 | Hypopharynx | Pyriform sinus | Never | Current, <10 pack-year | Never | Never | Negative | Negative |

*Tobacco chewing is light (if chewing index is <80) or heavy (if chewing index is ≥80); Tobacco chewing index is calculated as frequency of chewing event per day multiplied by duration in years; #A pack-year is defined as the equivalent of smoking one pack of bidis (25 bidis/pack) or one pack of cigarettes (10 cigarettes/pack) per day for 1 year..

| Table 3: Demographic details of the p16 positive (RNA-ISH negative) HNSCC patients | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sr No.** | **Sex** | **Age** | **Major tumor site** | **Tumor subsite** | **Tobacco chewing** | **Bidi smoking** | **Cigarette smoking** | **Alcohol use** | **HPV DNA-PCR** | **HPV RNA-ISH** |
| 1 | Male | 50 | Oropharynx | Base of tongue | Never | Current, >10 pack-year | Never | current | Negative | Negative |
| 2 | Male | 49 | Oropharynx | Base of tongue | Never | Former, >10 pack-year | Never | Never | Negative | Negative |
| 3 | Male | 44 | Oropharynx | Base of tongue | Current, heavy | Never | Never | Never | Negative | Negative |
| 4 | Male | 64 | Oropharynx | Base of tongue | Current, heavy | Never | Former, <10 pack-year | Former | Negative | Negative |
| 5 | Male | 54 | Oropharynx | Base of tongue | Former, light | Former, <10 pack-year | Never | Never | Negative | Negative |
| 6 | Male | 66 | Oropharynx | Soft palate | Never | Current, >10 pack-year | Never | current | Negative | Negative |
| 7 | Male | 65 | Oropharynx | Soft palate | Never | Current, >10 pack-year | Never | Never | Negative | Negative |
| 8 | Male | 59 | Oropharynx | Tonsil | Former, heavy | Former, <10 pack-year | Former, <10 pack-year | Never | Negative | Negative |
| 9 | Female | 35 | Larynx | Supraglottis | No info | No info | No info | No info | Negative | Negative |
| 10 | Male | 53 | Larynx | Supraglottis | Never | Current, <10 pack-year | Current, <10 pack-year | Never | Negative | Negative |
| 11 | Male | 52 | Larynx | Supraglottis | Former, light | Former, <10 pack-year | Never | Never | Negative | Negative |
| 12 | Male | 68 | Larynx | Supraglottis | Current, heavy | Current, <10 pack-year | Never | Current | Negative | Negative |
| 13 | Male | 59 | Hypopharynx | Pyriform sinus | Current, heavy | Current, >10 pack-year | Current, <10 pack-year | Current | Negative | Negative |
| 14 | Female | 59 | Hypopharynx | Pyriform sinus | Never | Current, <10 pack-year | Never | Never | Negative | Negative |
| 15 | Male | 56 | Hypopharynx | Pyriform sinus | Current, light | Current, <10 pack-year | Never | Current | Negative | Negative |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 16 | Male | 67 | Hypopharynx | Pyriform sinus | Current, heavy | Former, <10 pack-year | Never | Never | NA | Negative |
| 17 | Male | 57 | Hypopharynx | Pyriform sinus | Never | Current, >10 pack-year | Never | Never | Negative | Negative |
| 18 | Male | 30 | Hypopharynx | Pyriform sinus | Current, heavy | Current, <10 pack-year | Current, <10 pack-year | Current | Negative | Negative |
| 19 | Female | 61 | Hypopharynx | Pyriform sinus | Never | Never | Never | Never | Negative | Negative |
| 20 | Female | 64 | Hypopharynx | Post cricoid | Current, light | Never | Never | Never | Negative | Negative |

*Tobacco chewing is light (if chewing index is <80) or heavy (if chewing index is ≥80); Tobacco chewing index is calculated as frequency of chewing event per day multiplied by duration in years; #A pack-year is defined as the equivalent of smoking one pack of bidis (25 bidis/pack) or one pack of cigarettes (10 cigarettes/pack) per day for 1 year.

# List of Abbreviations

| | |
|---|---|
| AJCC | American joint committee on cancer's |
| ALDH1A1 | Aldehyde dehydrogenase 1A1 |
| ASR | Age standardized rate |
| CA9 | Carbonic anhydrase 9 |
| CAP | College of American pathologist |
| CD44 | Cluster of differentiation 44 |
| CD44v6 | Cluster of differentiation 44 variant 6 |
| CD98hc | Cluster of differentiation 98 heavy chain |
| CRC | Colorectal cancer |
| CSC | Cancer stem cell |
| DAB | Diaminobenzidine |
| DAPI | 4′,6-diamidino-2-phenylindole |
| dNTP | Deoxynucleoside triphosphate |
| DPX | Dibutylphthalate polystyrene xylene |
| ECOG | Eastern cooperative oncology group |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| FDA | Food and drug administration |
| FISH | Fluorescence in situ hybridization |
| FMISO | Fluoromisonidazole |
| H2O2 | Hydrogen peroxide |
| HA | hyaluronic acid |
| HIF1α | Hypoxia inducible factor 1α |

| | |
|---|---|
| HNS | Head and neck cancer |
| HNSCC | Head and neck squamous cell carcinoma |
| HPV | Human papilloma virus |
| ICD | International classification of diseases |
| IgG | Immunoglobulin G |
| IHC | Immunohistochemistry |
| IQR | Inter quartile range |
| LA-HNSCC | Locally advanced head and neck squamous cell carcinoma |
| LRC | Loco-regional control |
| mAb | Monoclonal antibody |
| MAPK | Mitogen activated protein kinase |
| MgCl2 | Magnesium chloride |
| mTOR | Mechanistic target of rapamycin |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NSCLC | Non-small cell lung cancers |
| OCT4 | Octamer-binding transcription factor 4 |
| OPSCC | Oropharyngeal squamous cell carcinoma |
| OS | Overall survival |
| PBS | Phosphate buffer saline |
| pEGFR | Phospho epidermal growth factor receptor |
| PET/CT | Positron emission tomography/computed tomography |
| PFS | Progression free survival |
| PI3K | Phosphatidylinositol 3-kinase |
| pRB | Retinoblastoma protein |
| REMARK | Reporting recommendations for tumor marker prognostic studies |

| | |
|---|---|
| RNA-ISH | RNA in-situ hybridization |
| SOX2 | Sex-determining region-Y homeobox-2 |
| SPSS | Statistical package for the social sciences |
| TKIs | Tyrosine kinase inhibitors |
| Tris | Trisaminomethane |
| UBC | Ubiquitin C |
| UICC | Union for international cancer control |
| URR | Upper regulatory region |
| VEGF | Vascular endothelial growth factor |

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