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The COMPLETE trial: HolistiC early respOnse assessMent for oroPharyngeaL cancer paTiEnts; Protocol for an observational study.

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ABSTRACT

Introduction

The incidence of oropharyngeal squamous cell carcinoma (OPSCC) continues to increase and despite improvements of several treatment strategies in the last decades, the locoregional failure (LRF) rate remains disappointingly high. This is especially the case in human papilloma virus (HPV) negative OPSCC, which has a two years LRF rate of 37%. In patients that do remain disease-free, long-term toxicity is substantial, severely impacting quality of life. Response prediction prior to or early during treatment, to identify poor and good responders, would provide opportunities for personalized treatment. However, within the OPSCC patient population no accurate predictive models are available for correct patient selection. Apparently, pivotal driving forces that determine how a tumor responds to treatment, are not yet elucidated. Therefore, this study focuses on a holistic approach to gain insight in novel potential prognostic biomarkers, acquired before and early during radiation treatment, to predict response to treatment in HPV-negative OPSCC patients.

Methods and analysis

This single-center prospective observational study investigates possible prognostic factors in 60 patients (age ≥ 18 yr) with histologically proven cT1-2N1-3M0 or cT3-4N0-3M0 HPV-negative OPSCC scheduled for primary radiotherapy with chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab) according to current clinical practice. To paint a complete picture of tumor response, a holistic approach will be used that aims to map the macroscopic, microscopic, and molecular landscape of the tumor before and during treatment. The macroscopic landscape will be assessed with Intra Voxel Incoherent Motion Diffusion Kurtosis Imaging (IVIM-DKI) acquired before, during, and three months after treatment; the microscopic landscape with biopsies of the primary tumor acquired before treatment and irradiated *ex vivo* to assess radiosensitivity; and the molecular landscape with circulating tumor DNA (ctDNA) analyzed before, during, and three months after treatment to assess the prevalence and evolution of tumor-specific genetic aberrations in the blood.

The main endpoint is locoregional control (LRC) two years after treatment, determined by clinical examination. The primary objective, focused on the macroscopic landscape only, is to determine whether a relative change in the mean of the diffusion coefficient *D* (an IVIM-DKI parameter) in the primary tumor early during treatment, improves the performance of a predictive model consisting of tumor volume only, for two years LRC after treatment. The secondary objectives are (1) to determine whether a relative change in mean diffusion coefficient *D* early during treatment improves the ability of this model, to predict the three months response after treatment; (2) to determine if other IVIM-DKI parameters, ctDNA, *ex vivo* sensitivity characteristics, and combinations thereof can be identified as potential novel prognostic markers, using an explorative analysis (3) to build a repository of imaging and ctDNA data to allow future identifications of biomarkers of treatment response.

Ethics and dissemination

The study was approved by the Medical Ethical Committee of Erasmus Medical Center. The main results of the trial will be presented in international meetings and medical journals.

Trial registration number NL8458 (www.trialregister.nl).

Strengths and limitations of this study

- This trial aims at developing an accurate predictive model for HPV-negative oropharyngeal squamous cell carcinoma patients.
- Early tumor response is assessed from macroscopic, microscopic, and molecular perspectives using a combination of novel MRI, *ex vivo* radiosensitivity and ctDNA techniques.
- A homogeneous patient population with only HPV-negative oropharyngeal squamous cell carcinoma will be studied.
- A repository of imaging and biological data will be created for future research.

- The study lacks statistical power to answer all secondary research objectives and therefore the secondary analyses are explorative.

INTRODUCTION

Head and neck cancer (HNC) is the sixth most common type of cancer worldwide with an estimated annual burden of 633,000 new cases and 355,000 deaths (1). Despite recent advances in treatment resulting in a better outcome for diseases such as melanoma or lung, the treatment results of HNC continue to disappoint, especially for human papilloma virus (HPV) negative head and neck cancer, while the incidence of HNC is increasing. Blanchard reported a two years overall survival (OS) of 50.7% for the chemoradiotherapy (CRT) group and 46.0% after radiotherapy (RT) alone in his meta analyses on HPV-negative oropharyngeal squamous cell carcinoma (OPSCC) (2). Ang et al., reported a three years locoregional recurrence rate (LRR) of 35.1% in the HPV-negative OPSCC group (3). This means that one third of the HPV-negative patients will probably die of a LRR. Furthermore, not only the outcome in this patient group is poor, but the burden of acute and late side effects is still substantial despite the introduction of modern radiation techniques (4-7).

Currently, 650 new patients with OPSCC are diagnosed annually in the Netherlands of which 40-50% are HPV-negative. If we could predict treatment response in this patient group before or early during treatment, this would open the door to clinical trials in which a more personalized treatment could be investigated; *e.g.* intensified (or in contrast, for those with poorer performance status, palliative therapy) for poor responders, and possibly less intense and thereby a less toxic therapy for good responders.

Although there have been studies performed to determine prognostic factors for HNC patients (8-13), to date no accurate predictive model exists for HPV-negative OPSCC patients for a number of reasons.

(1) Most previous studies have focused mainly on patient/clinical characteristics (tumor volume, age, smoking history, comorbidities) in addition to biomarkers of maximum one modality (*e.g.* MRI), while the response of the tumor depends on its entire, complex, multi-layered landscape (14). (2) Most studies focused on pre-treatment characteristics only, while a tumor is a dynamic system that changes during treatment. (3) Most studies were too small (N ~30) and contained patients with different types of head and neck tumors and both HPV-negative and HPV-positive tumors.

The current study was designed to overcome the abovementioned shortcomings by (1) studying the entire multilayered tumor landscape based on novel techniques focusing on the macroscopic, microscopic, and molecular landscape and (2) assessing changes in the tumor landscape early during treatment (3) in a patient cohort containing 60 patients with HPV-negative OPSCC patients only.

The *macroscopic* landscape will be studied with multi b-value diffusion-weighted imaging (DWI) using the hybrid Intra Voxel Incoherent Motion Diffusion Kurtosis Imaging (IVIM-DKI) model (15, 16). There is substantial data supporting that DWI is a promising tool for response assessment of HNC (17, 18). Obtaining additional parameters from DWI by employing Intra Voxel Incoherent Motion (IVIM) and diffusion kurtosis imaging (DKI) will enlarge the potential of macroscopic response prediction. This multi b-value DWI sequence will be obtained before and during treatment to study changes over time and will be corrected for artifacts (19, 20).

For the *microscopic* landscape, *ex vivo* radiosensitivity assessment of patient specific tumor biopsies will be obtained before treatment as potential biomarker of clinical outcome. We recently adapted our breast cancer organotypic tumor tissue slice method to be suitable for head and neck tumor tissue (publication in preparation) and developed a protocol for *ex vivo* radiation treatment of tumor

tissue (21). Using this method, tumor sensitivity to irradiation can be assessed for each individual patient.

Finally, the *molecular* landscape will be studied by analyzing liquid biopsies collecting circulating tumor DNA (ctDNA) for molecular tumor characteristics before and during treatment. Liquid biopsies are a promising minimal invasive alternative for tissue biopsies and serial samples at different time points during treatment are easily acquired. ctDNA comprises of DNA fragments derived from tumor cells, which enter the bloodstream after apoptosis or by active shedding of DNA fragments by living tumor cells. Genetic aberrations, such as mutations, can be identified and tracked in ctDNA, and correlated with clinical outcomes. In several tumor types, ctDNA detected at baseline and its evolution during treatment were shown to be strong prognostic factors (22-24). Wang et al. were able to detect ctDNA in plasma of HNC in a proof of principle study. In a small subgroup that did not develop tumor recurrence, no mutations were present shortly after primary surgery (25). This makes the detection of ctDNA a potential early biomarker that can be used to further tailor treatment.

METHODS AND ANALYSIS

Design and study population

The COMPLETE study is a single-center prospective observational study. In the period of August 2020 until August 2024, sixty patients will be included with histologically proven cT1-2N2-3M0 or cT3-4N0-3M0 HPV-negative OPSCC treated with primary radiotherapy and chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab).

Study objectives

Primary objective

Among the biomarker modalities explored in the current study (DWI, *ex vivo* radiosensitivity and ctDNA), most data is available on DWI parameters in relation to treatment outcome. Therefore, the primary objective of the study will be to determine if a relative change in the mean of the diffusion coefficient *D* (as obtained from IVIM-DKI) in the primary tumor early during treatment improves the performance of a predictive model consisting of only tumor volume for the two years locoregional control (LRC) after treatment of HPV-negative OPSCC patients.

Secondary objectives

1. To determine if a relative change in the mean of the diffusion coefficient *D* in the primary tumor early during treatment improves the performance of a predictive model including tumor volume only for the three months response after treatment of HPV-negative OPSCC patients.

2. To determine if other IVIM-DKI parameters (perfusion fraction *f*, pseudo-diffusion coefficient *D**, and kurtosis *K*), ctDNA, *ex vivo* radiosensitivity characteristics, and combinations thereof can be identified as a potential novel predictive markers for treatment response of HPV-negative OPSCC patients, using an explorative approach.

3. To build a repository of imaging data and liquid biopsies to allow future identifications of biomarkers of treatment response of HPV-negative OPSCC patients.

Inclusion criteria

- Patients with histologically proven cT1-2N1-3M0 or cT3-4N0-3M0 HPV-negative OPSCC
- Eighteen years or older
- Current and/-or former smoker
- Scheduled for primary radiotherapy with chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab)
- Standard planning MRI (including IVIM-DKI) successfully acquired
- Included in the BIO-ROC study (see Appendix 1 for details)
- Written informed consent

Exclusion criteria

- Patients with recurrence of previously confirmed head and neck squamous cell carcinoma or with other malignancies within the last five years
- Patients with previous irradiation or operation in a head and neck region overlapping with the current tumor
- Patients with any physical or mental status that interferes with the informed consent procedure or study procedures
- Patients with contraindications for MRI (*e.g.* claustrophobia, arterial clips in central nervous system)
- Patients with contraindications for Gadolinium contrast *(i.e.* hyper-sensitivity for Gadolinium or an impaired kidney function)

We will continue inclusion until we have 60 evaluable subjects, *i.e.* with the required MRI scans and blood samples .

Study procedures

The general outline of the study procedures is presented in Figure 1. Patients will be discussed in the weekly meeting of the multidisciplinary head and neck tumor board and patients will be treated according to the current clinical protocols. Patients will receive 70 Gy Intensity Modulated Radiotherapy (IMRT) or Intensity Modulated Proton beam Therapy (IMPT) in 35 fractions combined with cisplatin (100 mg/m^2 body-surface area (BSA), q3w or 40 mg/m^2 BSA, q1w) or cetuximab (initial dose of 400 mg/m^2, followed by 250 mg/m^2 weekly, for the duration of radiotherapy).

Timing of study procedures

Eligible patients are asked to participate in the BIO-ROC study (see Appendix 1). As part of the BIO-ROC study, a study specific biopsy, and a blood sample of 30 ml will be obtained before the start of treatment. An MRI scan will be performed before the start of treatment as part of standard work up. In the second week of treatment, a blood sample will be acquired for ctDNA analysis and the patient will undergo a second MRI scan. Three months after the completion of RT, at the time of clinical response evaluation, a third blood sample will be acquired for ctDNA analysis and the patient will undergo a third MRI scan.

The macroscopic landscape: IVIM-DKI

The MRI scans will be acquired with the patient immobilized in the radiotherapy mask. The MRI scan protocol consists of T1-weighted DIXON after Gadolinium contrast material injection, a T2-weighted TSE, a multi b-value DWI scan, and a DWI scan with inverse phase encoding gradient polarity for the

purpose of distortion correction. The multi b-value DWI scan consists of 15 b-values (0, 10, 2x80, 130, 570, 2x770, 2x780, 790, and 4x1500 s/mm^2) acquired in three orthogonal diffusion directions (20).

The microscopic landscape: Biopsy

For patients with a tumor that is accessible during physical examination (with or without histological confirmation), a tumor biopsy will be obtained by a head and neck surgeon during the outpatient clinic visit, within the frame of the BIO-ROC study (see Appendix 1). For patients without histology confirmed OPSCC, and requiring general anesthesia for proper tumor approach, two biopsies will be obtained during a single procedure, one for the diagnosis and one for the purpose of the study.

The molecular landscape: ctDNA blood samples

The blood sample of 30 mL for ctDNA analysis will be stored in CellSave tubes for ctDNA analysis at room temperature until processing it to plasma. Subsequently, cell-free DNA (cfDNA) will be isolated using the manual QIAmp circulating nucleic acid kit (Qiagen) or the automated QIAsymphony (Qiagen) or Maxwell kits (Promega). The plasma and isolated cfDNA will be stored at -80˚ and -30˚, respectively, until further analysis.

Patient follow up

Patients are monitored by the head and neck multidisciplinary team according to national guidelines. Follow-up visits will be planned every two months for the first year following RT. Starting from the second year, the frequency gradually decreases to every six months for a minimum of five years. LRC at two years will be determined by clinical examination and in case of doubt additional imaging and/ or biopsies will be acquired according to current clinical practice.

Data processing and analysis

The macroscopic layer: IVIM-DKI analysis

First, the multi-b-value DWI acquisitions will be corrected for geometric distortion and, if applicable, motion. To calculate the change in diffusion coefficient *D* between pre-treatment and early treatment, the DWI scans from week two will be registered to the DWI scans prior to treatment. The primary tumor will be delineated on the pretreatment T1w and T2w scan. Subsequently, the T1w and T2w scans are registered to the b=0 s/mm2 images of the DWI scan pretreatment. The diffusion coefficient values are calculated for each voxel in the ROI by fitting the IVIM-DKI model based on different b-values from the multi-b-value DWI acquisition:

$$S_i = S_0((1-f)\left(e^{-b_iD+\frac{1}{6}(b_iD)^2K}\right)+fe^{-b_iD^*})$$

where S_i is the measured signal intensity at the corresponding b-value b_i and S_0 the signal intensity at b-value of 0 s/mm^2, *D* the diffusion coefficient, *f* the perfusion fraction, *D** the pseudo-diffusion coefficient, and *K* the kurtosis. The b-values represent the amount of diffusion weighting. The mean diffusion coefficient *D* of the ROIs will be calculated for both the pre-treatment scans (acquired as part of the clinical protocol) and the scans acquired in the second week of treatment. The change in mean diffusion coefficient *D* during treatment compared to pretreatment is calculated and used for the statistical analysis of the primary endpoint. For each parameter, the distribution within the tumor is calculated. From the distribution, a large variety of metrics will be extracted, amongst others the standard deviation, and the 80th, 90th, 95th, and 99th percentiles, which will be used as input for an exploratory analysis. Moreover, supervoxels will be created to analyze the heterogeneity in the tumor.

The microscopic layer: *ex vivo* radiation and radiosensitivity testing

The tumor biopsies will be sliced into 300 µM thick slices and irradiated *ex vivo* with a single dose of 2 Gy, 5 Gy, or 7 Gy and cultured for five days. The percentage of proliferating cells of the irradiated tumor slices will be compared to untreated tumor slices after five days of culture. Proliferation will be detected by EdU incorporation and obtained microscopy images will be analyzed using in-house image processing software (Apoptosis Quantifier) for semi-automated quantification of the results. Similarly, increase in apoptosis in irradiated slices will be assessed after five days, using TUNEL staining. Untreated slices will be used as a control. The same in-house processing software will be used for microscopy image analysis. The outcomes of both assays will be analyzed as a continuous variable in the exploratory statistical analysis. Change in both parameters compared to the control will be used to describe tumor irradiation sensitivity.

The molecular layer: ctDNA analysis

A targeted approach with molecular barcoding will be applied using a panel of somatic genetic variations, based on the commercially available Oncomine™ Lung cfDNA assay. This panel covers eleven genes and >150 hotspots frequently mutated in non-small cell lung cancer (*ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1* and *TP53*). By measuring TP53 and the additional genes in the lung panel, we expect to cover most of the genetic aberrations of interest in HPV-negative OPSCC.

At least 20 ng of cfDNA will be sequenced using the above customized panel with molecular barcoding on the Ion Torrent NGS platform. The molecular barcoding will enable molecule quantification and detect mutations as low as 0.1% allele mutation frequency when evaluating 20 ng of cfDNA input. The TorrentSuite variant calling pipeline is used to identify tumor-specific variants for ctDNA detection, including *TP53* variants, and quantify the number of reads and independent

molecules with wild-type and variant sequence. Subsequently, based on these reads and molecule levels, the variant allele frequency (VAF) and the number of mutant molecules per mL blood will be established. DNA from the buffy coat will also be isolated and sequenced with this panel, to identify germline variants and mutations due clonal hematopoiesis.

The ctDNA extraction and analysis will be performed on the blood samples acquired pretreatment, acquired in the second week of treatment, and acquired at three months post-treatment. The change in the total number of mutant molecules in week two compared to baseline, specific genetic variants, the total number of mutations, the total ctDNA concentration in the blood and how these evolve during treatment will be described.

Statistical analyses

Primary objective

The dependent variable is LRC at two years (yes/no). The expected number of events in the trial is 22 (among 60 patients) which allows the testing of two explanatory variables based on the rule of thumb that ten events are required per variable.

A multivariable logistic regression will be performed with as dependent variable LRC at two years. Based on literature, tumor volume based on the delineated gross tumor volume is the most important variable associated with LRC two years after treatment among our patient population of only HPV-negative patients treated with primary radiotherapy with chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab) (8, 9, 26-28). The second variable that will be included is the relative change in mean diffusion coefficient *D* in week two compared to baseline as determined from the IVIM-DKI scans. The multivariable model including both parameters will be compared to the model without the change in mean diffusion coefficient *D*. A likelihood ratio test will be applied to

determine if the model with the change in mean diffusion coefficient *D* performs better than the model without; where a p-value < 0.05 will be considered statistically significant.

Secondary objectives

The first secondary objective is, apart from the endpoint at three months instead of two years, equivalent to the primary objective; the statistical analysis is therefore identical to the one described for the primary endpoint. The analysis for the first secondary objective will be performed once the three month endpoint is reached for all patients.

For the other secondary objectives, the parameters that will be analyzed include:

- Clinical/patient characteristics such as age, comorbidities, clinical tumor stage;
- IVIM-DKI parameters *D*, *f*, *D**, and *K* and their distributions within the tumor (at baseline and in week 2). Moreover, supervoxels will be generated based on the combination of *D*, *f*, *K* and *D** to investigate the effect of different distinct tumor regions on LRC;
- The established *ex vivo* radiosensitivity parameters (changes in proliferation and apoptosis upon irradiation with different irradiation doses);
- ctDNA parameters such as the total number of mutant molecules, the presence of specific genetic variants, the total ctDNA concentration in the blood and how these evolve during treatment.

Different endpoints will be considered: LRC at three months, LRC at two years and OS at two years.

Given the large number of variables compared to the number of events, conventional statistics are not suitable for the secondary objectives at this stage. Instead, an exploratory analysis will be performed using Least Absolute Shrinkage Selector Operator (LASSO) logistic regression. LASSO

logistic regression is a type of regression that shrinks the coefficients of the variables to avoid overfitting, while performing feature selection at the same time.

Given a large number of potentially interesting prognostic variables, feature selection is necessary but the risk of overfitting is significant. For the current dataset with relative few events, LASSO is a good balance between conventional statistical approaches, such as backward selection, and more black-box, data driven machine learning techniques. Analysis will be performed with the penalized package in R Statistical software. We will use L1 regularization given the large number of variables tested. Internal validation will be performed with cross-validation.

PATIENT AND PUBLIC INVOLVEMENT

The Dutch patient association for head and neck cancer (PVHH) gave feedback on our project during the development phase and will continue to provide feedback during the trial.

ETHICS AND DISSEMINATION

The COMPLETE trial is registered in Trialregister.nl (NL8458). The study was approved by the Medical Ethical Committee of Erasmus Medical Center (MEC 2020-0208).

PERSPECTIVE/ CONCLUSION

Although several new strategies implemented in the treatment of OPSCC patients have resulted in a better LRC, there is still an urgent need for improvement, especially for HPV-negative OPSCC patients. To be able to select the right patient for treatment intensification or de-intensification, an accurate predictive model needs to be developed. This predictive model should be based not only on patient or clinical characteristics but also on information of all layers of the tumor. Furthermore, these characteristics will have to be acquired on different time points (before and during treatment) to be able to take into account the dynamic process of the tumor over time. The COMPLETE trial aims at a holistic approach to assess the entire tumor landscape; at a macroscopic, microscopic, as well as molecular level. In a subsequent trial, these data can be used to design more personalized treatment strategies in patients with HPV-negative OPSCC to improve outcome.

Appendix 1.

Ancillary study: The BIO-ROC (BIOmarker of treatment Response in Oropharyngeal Cancer) study

All newly diagnosed OPSCC patients in our medical center are asked to participate in the BIO-ROC study that aims to assess the influence of intrinsic tumor properties on the treatment outcomes. This study is a prospective exploratory cohort study for OPSCC patients treated with primary radiotherapy with or without the addition of chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab) with curative intent. The goal is to assess the correlation between tumor *ex vivo* radiosensitivity with clinical response and to build a database of tumor and blood samples for future biomarker identification. For patients with OPSCC accessible during physical examination an additional tumor biopsy will be obtained pretreatment. For patients without histological confirmation of OPSCC and requiring general anesthesia for tumor approach, an extra biopsy next to the diagnostic one will be

obtained during a single procedure. For all patients an additional blood sample will be obtained pretreatment, at the end of week 2 during RT, and three months after RT during the clinical response evaluation visit. Clinical outcomes will be assessed within the standard follow-up scheme. In case of tumor recurrence, patients will be approached for obtaining additional tumor and blood samples. Additional informed consent will be asked for the BIO-ROC patients that meet the inclusion criteria of the COMPLETE protocol.

AUTHORS' CONTRIBUTIONS

Gerda M. Verduijn: designed the study protocol and wrote the manuscript. Marta E. Capala: provided input to the study protocol regarding the ex vivo radiation and radiosensitivity testing, and reviewed the manuscript. Nienke D. Sijtsema: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript. Iris Lauwers: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript. Juan A. Hernandez Tamames: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript, Wilma D. Heemsbergen: provided input to the study protocol regarding the study design and reviewed the manuscript. Aniel Sewnaik: provided input to the study protocol regarding the biopsy acquisition and reviewed the manuscript, Jose A. Hardillo: provided input to the study protocol regarding the biopsy acquisition and reviewed the manuscript. Hetty Mast: provided input to the study protocol regarding the biopsy acquisition and reviewed the manuscript. Yvette van Norden: provided input to the study protocol regarding the statistical analyses and reviewed the manuscript. Maurice P.H.M. Jansen: provided input to the study protocol regarding the ctDNA analysis and reviewed the manuscript. Aad van der Lugt: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript. Dik C. van Gent: provided input to the study protocol regarding the ex vivo radiation and radiosensitivity testing, and reviewed the manuscript. Mischa S. Hoogeman: provided input to the

study protocol regarding the study design and reviewed the manuscript. Bianca Mostert: provided input to the study protocol regarding the ctDNA analysis and reviewed the manuscript. Steven F. Petit: designed the study protocol and reviewed the manuscript.

COMPETING INTEREST STATEMENT

The department of radiotherapy has research collaborations with Elekta AB, Stockholm, Sweden and with Accuray Inc., Sunnyvale, CA, USA and Varian, Palo Alto, CA, USA.

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DATA SHARING STATEMENT

Data and results not yet generated

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893-917.

2. Blanchard P, Baujat B, Holostenco V, Bourredjem A, Baey C, Bourhis J, et al. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): a comprehensive analysis by tumour site. Radiother Oncol. 2011;100(1):33-40.

3. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363(1):24-35.

4. Wan Leung S, Lee TF, Chien CY, Chao PJ, Tsai WL, Fang FM. Health-related quality of life in 640 head and neck cancer survivors after radiotherapy using EORTC QLQ-C30 and QLQ-H&N35 questionnaires. BMC cancer. 2011;11:128.

5. Hunter KU, Schipper M, Feng FY, Lyden T, Haxer M, Murdoch-Kinch CA, et al. Toxicities affecting quality of life after chemo-IMRT of oropharyngeal cancer: prospective study of patient-reported, observer-rated, and objective outcomes. Int J Radiat Oncol Biol Phys. 2013;85(4):935-40.

6. Verdonck-de Leeuw IM, Buffart LM, Heymans MW, Rietveld DH, Doornaert P, de Bree R, et al. The course of health-related quality of life in head and neck cancer patients treated with chemoradiation: a prospective cohort study. Radiother Oncol. 2014;110(3):422-8.

7. Van den Bosch L, van der Laan HP, van der Schaaf A, Oosting SF, Halmos GB, Witjes MJH, et al. Patient-Reported Toxicity and Quality-of-Life Profiles in Patients With Head and Neck Cancer Treated With Definitive Radiation Therapy or Chemoradiation. Int J Radiat Oncol Biol Phys. 2021;111(2):456-67.

8. Linge A, Lohaus F, Löck S, Nowak A, Gudziol V, Valentini C, et al. HPV status, cancer stem cell marker expression, hypoxia gene signatures and tumour volume identify good prognosis subgroups in patients with HNSCC after primary radiochemotherapy: A multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). Radiother Oncol. 2016;121(3):364-73.

9. Knegjens JL, Hauptmann M, Pameijer FA, Balm AJ, Hoebers FJ, de Bois JA, et al. Tumor volume as prognostic factor in chemoradiation for advanced head and neck cancer. Head Neck. 2011;33(3):375-82.

10. Lassen P, Lacas B, Pignon JP, Trotti A, Zackrisson B, Zhang Q, et al. Prognostic impact of HPV-associated p16-expression and smoking status on outcomes following radiotherapy for oropharyngeal cancer: The MARCH-HPV project. Radiother Oncol. 2018;126(1):107-15.

11. Rietbergen MM, Brakenhoff RH, Bloemena E, Witte BI, Snijders PJ, Heideman DA, et al. Human papillomavirus detection and comorbidity: critical issues in selection of patients with oropharyngeal cancer for treatment De-escalation trials. Ann Oncol. 2013;24(11):2740-5.

12. Rietbergen MM, Witte BI, Velazquez ER, Snijders PJ, Bloemena E, Speel EJ, et al. Different prognostic models for different patient populations: validation of a new prognostic model for patients with oropharyngeal cancer in Western Europe. Br J Cancer. 2015;112(11):1733-6.

13. van der Schroeff MP, Steyerberg EW, Wieringa MH, Langeveld TP, Molenaar J, Baatenburg de Jong RJ. Prognosis: a variable parameter: dynamic prognostic modeling in head and neck squamous cell carcinoma. Head Neck. 2012;34(1):34-41.

14. Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nat Rev Cancer. 2018;18(5):269-82.

15. Le Bihan D. What can we see with IVIM MRI? Neuroimage. 2019;187:56-67.

16. Lu Y, Jansen JF, Mazaheri Y, Stambuk HE, Koutcher JA, Shukla-Dave A. Extension of the intravoxel incoherent motion model to non-gaussian diffusion in head and neck cancer. J Magn Reson Imaging. 2012;36(5):1088-96.

17. Vandecaveye V, Dirix P, De Keyzer F, Op de Beeck K, Vander Poorten V, Hauben E, et al. Diffusion-weighted magnetic resonance imaging early after chemoradiotherapy to monitor treatment response in head-and-neck squamous cell carcinoma. Int J Radiat Oncol Biol Phys. 2012;82(3):1098-107.

18. King AD, Chow KK, Yu KH, Mo FK, Yeung DK, Yuan J, et al. Head and neck squamous cell carcinoma: diagnostic performance of diffusion-weighted MR imaging for the prediction of treatment response. Radiology. 2013;266(2):531-8.

19. Poot DH, den Dekker AJ, Achten E, Verhoye M, Sijbers J. Optimal experimental design for diffusion kurtosis imaging. IEEE Trans Med Imaging. 2010;29(3):819-29.

20. Sijtsema ND, Petit SF, Poot DHJ, Verduijn GM, van der Lugt A, Hoogeman MS, et al. An optimal acquisition and post-processing pipeline for hybrid IVIM-DKI in head and neck. Magn Reson Med. 2021;85(2):777-89.

21. Naipal KA, Verkaik NS, Sanchez H, van Deurzen CH, den Bakker MA, Hoeijmakers JH, et al. Tumor slice culture system to assess drug response of primary breast cancer. BMC cancer. 2016;16:78.

22. Goldberg SB, Narayan A, Kole AJ, Decker RH, Teysir J, Carriero NJ, et al. Early Assessment of Lung Cancer Immunotherapy Response via Circulating Tumor DNA. Clinical cancer research : an official journal of the American Association for Cancer Research. 2018;24(8):1872-80.

23. Cheng H, Liu C, Jiang J, Luo G, Lu Y, Jin K, et al. Analysis of ctDNA to predict prognosis and monitor treatment responses in metastatic pancreatic cancer patients. Int J Cancer. 2017;140(10):2344-50.

24. van Ginkel JH, Slieker FJB, de Bree R, van Es RJJ, Van Cann EM, Willems SM. Cell-free nucleic acids in body fluids as biomarkers for the prediction and early detection of recurrent head and neck cancer: A systematic review of the literature. Oral Oncol. 2017;75:8-15.

25. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci Transl Med. 2015;7(293):293ra104.

26. Lok BH, Setton J, Caria N, Romanyshyn J, Wolden SL, Zelefsky MJ, et al. Intensity-modulated radiation therapy in oropharyngeal carcinoma: effect of tumor volume on clinical outcomes. Int J Radiat Oncol Biol Phys. 2012;82(5):1851-7.

27. Carpén T, Saarilahti K, Haglund C, Markkola A, Tarkkanen J, Hagström J, et al. Tumor volume as a prognostic marker in p16-positive and p16-negative oropharyngeal cancer patients treated with definitive intensity-modulated radiotherap. Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft [et al]. 2018;194(8):759-70.

28. Studer G, Lütolf UM, El-Bassiouni M, Rousson V, Glanzmann C. Volumetric staging (VS) is superior to TNM and AJCC staging in predicting outcome of head and neck cancer treated with IMRT. Acta Oncol. 2007;46(3):386-94.

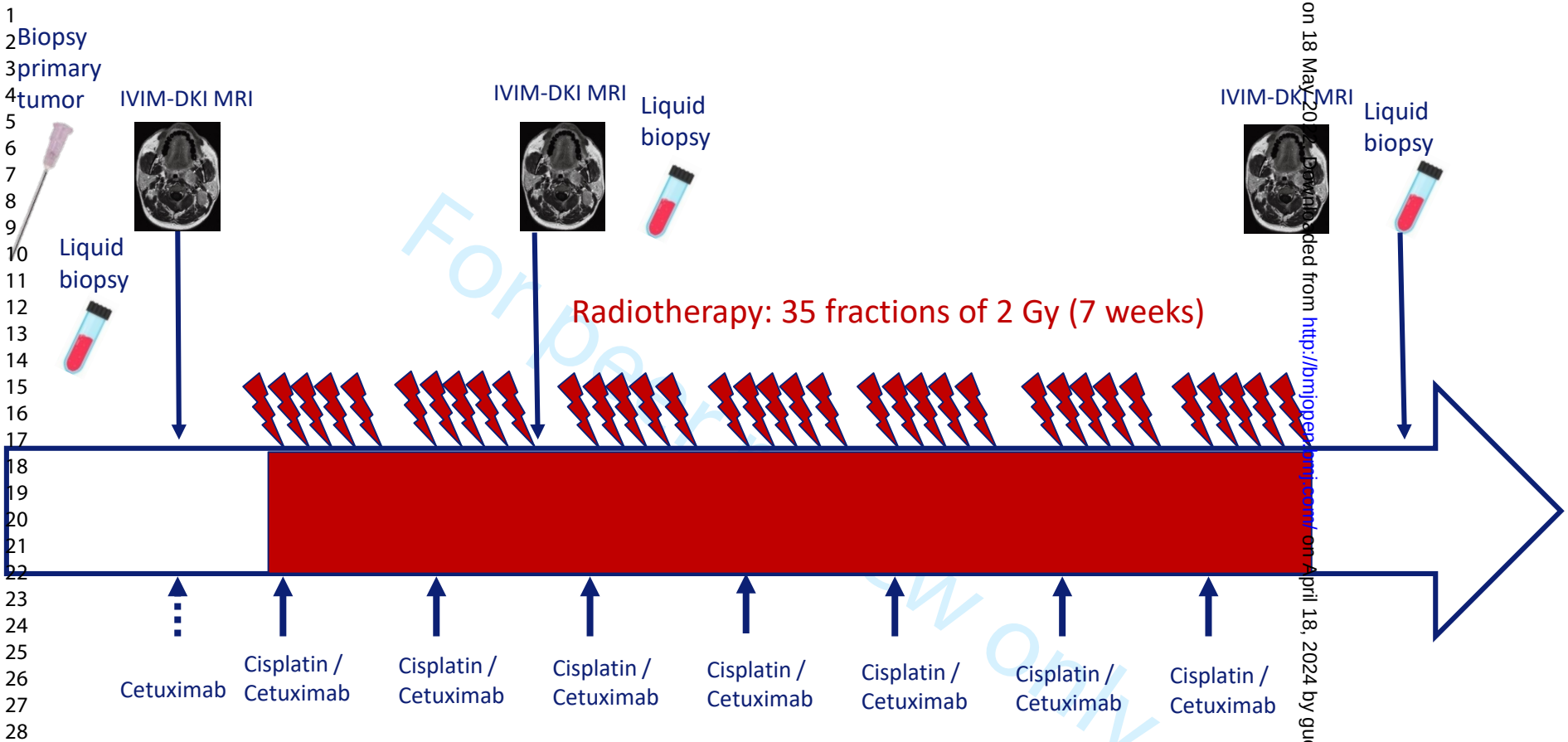

Figure 1. Study procedures of COMPLETE.

BMJ Open

The COMPLETE trial: HolistiC early respOnse assessMent for oroPharyngeaL cancer paTiEnts; Protocol for an observational study.

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The COMPLETE trial: HolistiC early respOnse assessMent for oroPharyngeaL cancer paTiEnts; Protocol for an observational study.

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ABSTRACT

Introduction

The locoregional failure (LRF) rate in HPV-negative oropharyngeal squamous cell carcinoma (OPSCC) remains disappointingly high and toxicity is substantial. Response prediction prior to or early during treatment, would provide opportunities for personalized treatment. Currently, there are no accurate predictive models available for correct OPSCC patient selection. Apparently, the pivotal driving forces that determine how a OPSCC responds to treatment, have yet to be elucidated. Therefore, the COMPLETE study focuses on a holistic approach to gain insight in novel potential prognostic biomarkers, acquired before and early during treatment, to predict response to treatment in HPV-negative OPSCC patients.

Methods and analysis

This single-center prospective observational study investigates 60 HPV-negative OPSCC patients scheduled for primary radiotherapy with cisplatin or cetuximab, according to current clinical practice. A holistic approach will be used that aims to map the macroscopic (with Intra Voxel Incoherent Motion Diffusion Kurtosis Imaging (IVIM-DKI); before, during, and three months after RT), microscopic (with biopsies of the primary tumor acquired before treatment and irradiated *ex vivo* to assess radiosensitivity), and molecular landscape (with circulating tumor DNA (ctDNA) analyzed before, during, and three months after treatment). The main endpoint is locoregional control (LRC) two years after treatment. The primary objective is to determine whether a relative change in the mean of the diffusion coefficient *D* (an IVIM-DKI parameter) in the primary tumor early during treatment, improves the performance of a predictive model consisting of tumor volume only, for two years LRC after treatment. The secondary objectives investigate the potential of other IVIM-DKI parameters, *ex vivo* sensitivity characteristics, ctDNA, and combinations thereof as potential novel prognostic markers.

Ethics and dissemination

The study was approved by the Medical Ethical Committee of Erasmus Medical Center. The main results of the trial will be presented in international meetings and medical journals.

Trial registration number NL8458 (www.trialregister.nl).

Strengths and limitations of this study

- In this trial a predictive model for HPV-negative oropharyngeal squamous cell carcinoma patients will be developed.
- Early tumor response is assessed from macroscopic, microscopic, and molecular perspectives using a combination of novel MRI (IVIM-DKI), *ex vivo* radiosensitivity, and ctDNA techniques.
- A homogeneous patient population with only HPV-negative oropharyngeal squamous cell carcinoma is included.
- The primary objective, focuses on the change in mean diffusion coefficient early during treatment.
- The analysis of the secondary objectives is explorative, due to sample size restrictions.

INTRODUCTION

Head and neck cancer (HNC) is the sixth most common type of cancer worldwide with an estimated annual burden of 633,000 new cases and 355,000 deaths (1). Despite recent advances in treatments resulting in better outcomes for diseases such as melanoma or lung cancer, the treatment of HNC continues to disappoint, especially for human papilloma virus (HPV)-negative head and neck cancer. Blanchard et al., reported two years overall survival (OS) of 50.7% for the chemoradiotherapy (CRT) group, and 46.0% after radiotherapy (RT) alone in his meta analyses on HPV-negative oropharyngeal squamous cell carcinoma (OPSCC) (2). Ang et al., reported three year locoregional recurrence rate (LRR) of 35.1% in the HPV-negative OPSCC group (3). This rate indicates, that a considerable number of patients die due to locoregional recurrence for which there are no other curative treatment options in the majority of cases. Furthermore, the burden of acute and late side effects is still substantial despite the introduction of modern radiation techniques (4-7).

Currently, 650 new patients with OPSCC are diagnosed annually in the Netherlands of which 40-50% are HPV-negative. If we could predict treatment response in this patient group before or early during treatment, this would open the door to clinical trials in which a more personalized treatment could be investigated; *e.g.* intensified (or in contrast, for those with poorer performance status, palliative therapy) for poor responders, and possibly less intense and thereby a less toxic therapy for good responders. Although there have been studies performed to determine prognostic factors for HNC patients (8-13), to date no accurate predictive model exists for HPV-negative OPSCC patients for a number of reasons. (1) Previous studies have focused mainly on patient/clinical characteristics (tumor volume, age, smoking history, comorbidities) in addition to biomarkers of maximum one modality (*e.g.* MRI), while the response of the tumor depends on its entire, complex, multi-layered landscape (14). (2) Many studies focused on pre-treatment characteristics only, while a tumor is a dynamic system that changes during treatment. (3) Studies are too small (N ~30) and contain

patients with different types of head and neck tumors as well as HPV-negative and HPV-positive tumors combined.

The current COMPLETE study was designed to address these shortcomings directly by; (1) Studying the entire multilayered tumor landscape based on novel techniques focusing on the macroscopic, microscopic, and molecular landscape. (2) Assess changes in the tumor landscape early during treatment; and (3) Acquire data in a cohort consisting of 60 patients with HPV-negative OPSCC, respectively.

The *macroscopic* tumor landscape will be studied with multi b-value diffusion-weighted imaging (DWI) using the hybrid Intra Voxel Incoherent Motion Diffusion Kurtosis Imaging (IVIM-DKI) model (15, 16). With DWI the extracellular movement of water molecules is detected and quantified by the apparent diffusion coefficient (ADC). When adding the IVIM-DKI model, perfusion and intracellular diffusion (reflected by the kurtosis), are taken into account. Obtaining additional parameters from DWI by employing Intra Voxel Incoherent Motion (IVIM) and diffusion kurtosis imaging (DKI) will enlarge the potential of macroscopic response prediction. This multi b-value DWI sequence will be obtained before, during, and after treatment to study changes over time (17, 18).

For the *microscopic* landscape, *ex vivo* radiosensitivity assessment of patient specific tumor biopsies will be obtained before treatment as a potential biomarker of clinical outcome. We recently adapted our breast cancer organotypic tumor tissue slice method to be suitable for head and neck tumor tissue (publication in preparation) and developed a protocol for *ex vivo* radiation treatment of tumor tissue (19). Using this method, tumor sensitivity to irradiation can be assessed for each individual patient.

Finally, the *molecular* landscape will be studied by analyzing liquid biopsies collecting circulating tumor DNA (ctDNA) for molecular tumor characteristics before, during, and after treatment. Liquid biopsies are a promising minimal invasive alternative for tissue biopsies and serial samples at different time points during treatment are easily acquired. ctDNA comprises of DNA fragments

derived from tumor cells, which enter the bloodstream after apoptosis or by active shedding of DNA fragments by living tumor cells. Genetic aberrations, such as mutations, can be identified and tracked in ctDNA, and correlated with clinical outcomes. In several tumor types, ctDNA detected at baseline and its evolution during treatment were shown to be strong prognostic factors (20-22). Wang et al. were able to detect ctDNA in plasma of HNC in a proof of principle study. In a small subgroup that did not develop tumor recurrence, no mutations were present shortly after primary surgery (23). This makes the detection of ctDNA a potential early biomarker that can be used to further tailor treatment.

METHODS AND ANALYSIS

Design and study population

The COMPLETE study is a single-center prospective observational study. In the period of August 2020 until August 2024, sixty patients will be included with histologically proven cT1-2N2-3M0 or cT3-4N0-3M0 HPV-negative OPSCC treated with primary radiotherapy and chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab). For the choice of number of patients we refer to the power calculation in the statistical section.

Study objectives

Primary objective

Among the biomarker modalities explored in the current study (DWI, *ex vivo* radiosensitivity, and ctDNA), most data is available on DWI parameters in relation to treatment outcome. Therefore, the primary objective of the study will be to determine if a relative change in the mean of the diffusion coefficient *D* (as obtained from IVIM-DKI) in the primary tumor early during treatment improves the performance of a predictive model consisting of only tumor volume for the two years locoregional control (LRC) after treatment of HPV-negative OPSCC patients.

Secondary objectives

1. To determine if a relative change in the mean of the diffusion coefficient *D* in the primary tumor early during treatment improves the performance of a predictive model including tumor volume only for the three months response after treatment of HPV-negative OPSCC patients.

2. To determine if other IVIM-DKI parameters (perfusion fraction *f*, pseudo-diffusion coefficient *D**, and kurtosis *K*), ctDNA, *ex vivo* radiosensitivity characteristics, and combinations thereof can be identified as a potential novel predictive markers for treatment response of HPV-negative OPSCC patients, using an explorative approach.

3. To build a repository of imaging data and liquid biopsies to allow future identifications of biomarkers of treatment response of HPV-negative OPSCC patients.

Inclusion criteria

- Patients with histologically proven cT1-2N1-3M0 or cT3-4N0-3M0 HPV-negative OPSCC
- Eighteen years or older
- Current and/-or former smoker

- Scheduled for primary radiotherapy with chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab)
- Standard planning MRI (including IVIM-DKI) successfully acquired
- Included in the BIO-ROC study (see Appendix 1 for details)
- Written informed consent

Exclusion criteria

- Patients with recurrence of previously confirmed head and neck squamous cell carcinoma or with other malignancies within the last five years
- Patients with previous irradiation or operation in a head and neck region overlapping with the current tumor
- Patients with any physical or mental status that interferes with the informed consent procedure or study procedures
- Patients with contraindications for MRI (*e.g.* claustrophobia, arterial clips in central nervous system)
- Patients with contraindications for Gadolinium contrast *(i.e.* hyper-sensitivity for Gadolinium or an impaired kidney function)

We will continue inclusion until we have 60 evaluable subjects, *i.e.* with the required MRI scans and blood samples .

Study procedures

The general outline of the study procedures is presented in Figure 1. Patients will be discussed in the weekly meeting of the multidisciplinary head and neck tumor board and patients will be treated according to the current clinical protocols. Patients will receive 70 Gy Intensity Modulated Radiotherapy (IMRT) or Intensity Modulated Proton beam Therapy (IMPT) in 35 fractions combined with cisplatin (100 mg/m^2 body-surface area (BSA), q3w or 40 mg/m^2 BSA, q1w) or cetuximab (initial dose of 400 mg/m^2, followed by 250 mg/m^2 weekly, for the duration of radiotherapy).

Timing of study procedures

Eligible patients are asked to participate in the BIO-ROC study (see Appendix 1). As part of the BIO-ROC study, a study specific biopsy, and a blood sample of 30 ml will be obtained before the start of treatment. An MRI scan will be performed before the start of treatment as part of standard work up. In the second week of treatment, a blood sample will be acquired for ctDNA analysis and the patient will undergo a second MRI scan. Three months after the completion of RT, at the time of clinical response evaluation, a third blood sample will be acquired for ctDNA analysis and the patient will undergo a third MRI scan.

The macroscopic landscape: IVIM-DKI

MRI scans will be acquired with the patient immobilized in treatment position (*i.e.* with radiotherapy mask). The MRI scan protocol consists of T1-weighted DIXON after Gadolinium contrast material injection, a T2-weighted TSE, a multi b-value DWI scan, and a DWI scan with inverse phase encoding gradient polarity for the purpose of distortion correction (flip angle: 90 degrees; TR: 6700 ms; TE 81.8 ms; FOV 26 x 26 cm; 4 mm slice thickness; 0.2 mm gap, 128 x 128 matrix; bandwith: 1953.12 Hz/ pixel). The multi b-value DWI scan consists of 15 b-values (0, 10, 2x80, 130, 570, 2x770,

2x780, 790, and 4x1500 s/mm^2) acquired in three orthogonal diffusion directions (18), where the b-values represent the amount of diffusion weighting.

The microscopic landscape: Biopsy

For patients with a tumor that is accessible during physical examination (with or without histological confirmation), a tumor biopsy will be obtained by a head and neck surgeon during the outpatient clinic visit according to the BIO-ROC study (see Appendix 1). For patients without histology confirmed OPSCC, and requiring general anesthesia for proper tumor approach, two biopsies will be obtained during a single procedure, one for the diagnosis and one for the purpose of the study. The tumor biopsies will be sliced into 300 µM thick slices and irradiated *ex vivo* and cultured for five days. Based on preliminary results from our laboratory, a single dose of 5 Gy resulted in the best discrimination between irradiation-sensitive and irradiation-resistant tumors (24). Therefore, all tumor biopsies (of individual patients) used in the current study, will be treated with a single dose of 5 Gy. In case more tumor material is available allowing for multiple treatment conditions, separate slices of the same tumor will also be treated with a single dose of 2 Gy or 7 Gy to gain more insight into the irradiation sensitivity of a given tumor.

The molecular landscape: ctDNA blood samples

Blood samples containing 30 mL blood for ctDNA analysis will be stored in CellSave tubes for ctDNA analysis at room temperature until processing it to plasma. Subsequently, cell-free DNA (cfDNA) will be isolated using the manual QIAmp circulating nucleic acid kit (Qiagen) or the automated QIAsymphony (Qiagen) or Maxwell kits (Promega). The plasma and isolated cfDNA will be stored at -80˚ and -30˚, respectively, until further analysis.

Patient follow up

Patients are monitored by the head and neck multidisciplinary team according to national guidelines. Follow-up visits will be planned every two months for the first year following RT. Starting from the second year, the frequency gradually decreases to every six months for a minimum of five years. LRC at two years will be determined by clinical examination and in case of doubt additional imaging and/ or biopsies will be acquired according to current clinical practice.

Data processing and analysis

The macroscopic layer: IVIM-DKI analysis

The primary tumor will be delineated on the pretreatment T1w and T2w scan. The multi-b-value DWI acquisitions will be processed according to Sijtsema et al (18). In short, first the scans for each b-value will be corrected for geometric distortion with FSL (FMRIB Software Library) (25, 26). Second, the scans of the individual b-values are registered rigidly to the scan with b=0 s/mm2. Note that a rigid registration is expected to suffice since patients are scanned with the RT mask. Then the region of interest (ROI), as defined by the primary tumor contours, is projected on top of the scan with b=0 s/mm2. Then the diffusion coefficient values are calculated for each voxel in the ROI by fitting the IVIM-DKI model based on different b-values from the multi-b-value DWI acquisition:

$$S_i = S_0((1-f)\left(e^{-b_iD+\frac{1}{6}(b_iD)^2K}\right)+fe^{-b_iD^*})$$

where S_i is the measured signal intensity at the corresponding b-value b_i and S_0 the signal intensity at b-value of 0 s/mm^2, D the diffusion coefficient, f the perfusion fraction, D^* the pseudo-diffusion coefficient, and K the kurtosis. The b-values represent the amount of diffusion weighting. The mean diffusion coefficient D of the ROIs will be calculated for both the pre-treatment scans (acquired as part of the clinical protocol) and the scans acquired in the second week of treatment. The percentage

change in mean diffusion coefficient *D* during treatment compared to pretreatment is used for the statistical analysis of the primary endpoint. Next, for *D, f, D*, and K* the distribution within the tumor is calculated. From the distribution, a large variety of metrics will be extracted, amongst others the standard deviation, and the 80th, 90th, 95th, and 99th percentiles, which will be used as input for an exploratory analysis. Moreover, supervoxels will be created to analyze the heterogeneity in the tumor.

The microscopic layer: *ex vivo* radiation and radiosensitivity testing

The percentage of proliferating cells of the irradiated tumor slices will be compared to untreated tumor slices after five days of culture. Proliferation will be detected by EdU incorporation and obtained microscopy images will be analyzed using in-house image processing software (Apoptosis Quantifier) for semi-automated quantification of the results. Similarly, increase in apoptosis in irradiated slices will be assessed after five days, using TUNEL staining. Untreated slices will be used as a control. The same in-house processing software will be used for microscopy image analysis. The outcomes of both assays will be analyzed as a continuous variable in the exploratory statistical analysis. Change in both parameters compared to the control will be used to describe tumor irradiation sensitivity.

The molecular layer: ctDNA analysis

A targeted approach with molecular barcoding will be applied using a panel of somatic genetic variations, including TP53, PIK3CA, CDKN2A, FBXW7, HRAS, NRAS, FAT1, and MOTCH1 (23, 27). This panel will be extended based on most recent available primary tumor sequencing data and literature at time of analysis, which will be expected to cover the relevant genetic aberrations of interest in HPV-negative OPSCC.

At least 20 ng of cfDNA will be sequenced using the above customized panel with molecular barcoding on the Ion Torrent NGS platform. The molecular barcoding will enable molecule quantification and detect mutations as low as 0.1% allele mutation frequency when evaluating 20 ng of cfDNA input. The TorrentSuite variant calling pipeline is used to identify tumor-specific variants for ctDNA detection, including *TP53* variants, and quantify the number of reads and independent molecules with wild-type and variant sequence. Subsequently, based on these reads and molecule levels, the variant allele frequency (VAF) and the number of mutant molecules per mL blood will be established. DNA from the buffy coat will also be isolated and sequenced with this panel, to identify germline variants and mutations due clonal hematopoiesis.

The ctDNA extraction and analysis will be performed on the blood samples acquired pretreatment, acquired in the second week of treatment, and acquired at three months post-treatment. The change in the total number of mutant molecules in week two compared to baseline, specific genetic variants, the total number of mutations, the total ctDNA concentration in the blood and how these evolve during treatment will be described.

Statistical analyses

Primary objective

The dependent variable is LRC at two years (yes/no). Based on relevant literature (10), within our study population of patients with HPV-negative oropharynx tumors and a smoking history, 37% of the patients are expected to have local tumor progression within 2 years (the primary outcome of interest). We expect to be able to include 60 patients in four years, which will lead to approximately 22 events in total. Twenty-two events allows the testing of two explanatory variables based on the rule of thumb that ten events are required per variable. In case of missing values, the analyses will be

done on the complete cases for the specific analysis but with sensitivity analyses after imputation on all included patients.

A multivariable logistic regression will be performed with as dependent variable LRC at two years. According to literature, tumor volume based on the delineated gross tumor volume pre-RT is the most important variable associated with LRC two years after treatment among our patient population of only HPV-negative patients treated with primary radiotherapy with chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab) (8, 9, 28-30). The second variable that will be included is the relative change in mean diffusion coefficient *D* in week two compared to baseline as determined from the IVIM-DKI scans. The multivariable model including both parameters will be compared to the model without the change in mean diffusion coefficient *D*. A likelihood ratio test will be applied to determine if the model with the change in mean diffusion coefficient *D* performs better than the model without; where a p-value < 0.05 will be considered statistically significant.

Secondary objectives

The first secondary objective is, apart from the endpoint at three months instead of two years, equivalent to the primary objective; the statistical analysis is therefore identical to the one described for the primary endpoint. The analysis for the first secondary objective will be performed once the three month endpoint is reached for all patients.

For the other secondary objectives, the parameters that will be analyzed include:

- Clinical/patient characteristics such as age, comorbidities, clinical tumor stage;
- IVIM-DKI parameters *D*, *f*, *D**, and *K* and their distributions within the tumor (at baseline and in week 2). Moreover, supervoxels will be generated based on the combination of *D*, *f*, *K,* and *D** to investigate the effect of different distinct tumor regions on LRC;

- The established *ex vivo* radiosensitivity parameters (changes in proliferation and apoptosis upon irradiation with different irradiation doses);
- ctDNA parameters such as the total number of mutant molecules, the presence of specific genetic variants, the total ctDNA concentration in the blood and how these evolve during treatment.

Different endpoints will be considered: LRC at three months, LRC at two years and OS at two years.

Given the large number of variables compared to the number of events, feature selection is necessary but the risk of overfitting is significant. As conventional statistics are not suitable for the secondary objectives, an exploratory analysis will be performed using Least Absolute Shrinkage Selector Operator (LASSO) logistic regression. LASSO logistic regression is a type of regression that shrinks the coefficients of the variables to avoid overfitting, while performing feature selection at the same time. Furthermore, LASSO is a good balance between conventional statistical approaches, such as backward selection, and more black-box, data driven machine learning techniques. Analysis will be performed with the penalized package in R Statistical software. We will use L1 regularization given the large number of variables tested. Internal validation will be performed with cross-validation. In correspondence to the primary hypothesis, in case of missing values, the analyses will be done on the complete cases for the specific analysis but with sensitivity analyses after imputation on all included patients.

PATIENT AND PUBLIC INVOLVEMENT

The Dutch patient association for head and neck cancer (PVHH) gave feedback on our project during the development phase and will continue to provide feedback during the trial.

ETHICS AND DISSEMINATION

The COMPLETE trial is registered in Trialregister.nl (NL8458). The study was approved by the Medical Ethical Committee of Erasmus Medical Center (MEC 2020-0208). The COMPLETE trial is supported by the Dutch patient association for head and neck cancer (PVHH). The methods and findings of the study will be published in peer-reviewed journals and presented on national and international conferences.

DISCUSSION

Although several strategies implemented in recent years in the treatment of OPSCC patients have increased LRC, there is still an urgent need for improvement, especially for HPV-negative OPSCC patients. To be able to select the right patient for treatment intensification or de-intensification, accurate predictive model needs to be developed. Given the complexity and the dynamics of tumor response as an interaction between the different 'layers' (macroscopic, microscopic, and molecular) that evolve as a result of treatment, we believe that for accurate prediction models the different layers and the dynamics of response should be incorporated. In the current COMPLETE study we aim to assess the entire multilayered tumor landscape based on novel techniques focusing on the macroscopic, microscopic, and molecular landscape before and early during treatment, in a patient cohort containing 60 patients with HPV-negative OPSCC patients only.

There is a delicate balance between acquiring as much information as possible before and during treatment, while limiting the number of procedures patients need to undergo. For the macroscopic data we chose to focus on the novel IVIM-DKI MRI technique, since conventional DWI has shown to be promising for response assessment of HNC (31-34). IVIM-DKI adds information compared to conventional DWI but also has limitations. For instance, Sijtsema et al. demonstrated a relative

repeatability coefficient of the diffusion coefficient *D* of 38% in healthy volunteers (18). So, fairly large changes in *D* need to occur to be detected as a true change, as small changes will be within normal measurements variation. As an alternative, several other functional imaging modalities could have been candidates to provide early response assessment as well for the macroscopic layer, *e.g.* FDG PET-CT (35). Our decision to focus on MRI, was based on prior studies (31, 32); that MRI is part of our standard workflow in RT planning for HNC, and therefore does not require an additional scanning session pretreatment; and the short scanning time resulting in manageable patient discomfort. Possibly, adding one or two PET-CT on top of the MRI scans would have provided additional interesting data, but was deemed infeasible regarding the additional patient burden.

For microscopic data, we study the response of tumor biopsies to irradiation *ex vivo*. This novel technique might have profound clinical implications, allowing individualized treatment of OPSCC patients. However, for several reasons, *ex vivo* response may not turn out to be representative for patient response. For instance, the biopsy may not represent intra-tumor heterogeneity of a tumor that may consists of different tumor regions. Furthermore, tumor tissue is grossly selected at the outpatient clinic without microscopic confirmation potentially yielding tissue with low cellularity. However, based on our experience so far, the risk of missampling is small.

For the molecular data we focus on ctDNA as this is a promising biomarker that is easily acquired (20-23). A possible limitation of ctDNA is the detection of DNA fragments at very low concentrations. Other possible candidates to assess the molecular landscape would have been circulating tumor cells (CTCs), miRNA, and cfRNA. However, since CTCs have so far not been established as a prognostic marker in locally advanced HNC and the low sensitivity in the primary (non-metastasized) setting, no CTCs analyses are part of the study (36). miRNAs are also a promising prognostic marker, but is not an area of expertise in our laboratory and was therefore not chosen as a marker. cfRNA as a biomarker is strongly challenged by the need to process blood samples quickly after blood draw, which is a challenge logistic-wise.

We expect that, given the complexity of tumor response, the holistic approach we propose is promising to identify combinations of biomarkers for accurate prediction models. Naturally, studying multiple variables has as important drawback the required number of events for sufficient statistical power. Therefore the study was powered solely on a macroscopic level parameter; the change in mean diffusion coefficient. The secondary objectives that combine multiple parameters from the different layers should be considered therefore as explorative and hypothesis generating to select high potential combination of biomarkers to be validated in subsequent trials.

AUTHORS' CONTRIBUTIONS

Gerda M. Verduijn: designed the study protocol and wrote the manuscript. Marta E. Capala: provided input to the study protocol regarding the ex vivo radiation and radiosensitivity testing, and reviewed the manuscript. Nienke D. Sijtsema: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript. Iris Lauwers: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript. Juan A. Hernandez Tamames: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript, Wilma D. Heemsbergen: provided input to the study protocol regarding the study design and reviewed the manuscript. Aniel Sewnaik: provided input to the study protocol regarding the biopsy acquisition and reviewed the manuscript, Jose A. Hardillo: provided input to the study protocol regarding the biopsy acquisition and reviewed the manuscript. Hetty Mast: provided input to the study protocol regarding the biopsy acquisition and reviewed the manuscript. Yvette van Norden: provided input to the study protocol regarding the statistical analyses and reviewed the manuscript. Maurice P.H.M. Jansen: provided input to the study protocol regarding the ctDNA analysis and reviewed the manuscript. Aad van der Lugt: provided input to the study protocol regarding the IVIM-DKI analysis and reviewed the manuscript. Dik C. van Gent: provided input to the study protocol regarding the ex vivo radiation and

radiosensitivity testing, and reviewed the manuscript. Mischa S. Hoogeman: provided input to the study protocol regarding the study design and reviewed the manuscript. Bianca Mostert: provided input to the study protocol regarding the ctDNA analysis and reviewed the manuscript. Steven F. Petit: designed the study protocol and reviewed the manuscript.

COMPETING INTEREST STATEMENT

The department of radiotherapy has research collaborations with Elekta AB, Stockholm, Sweden and with Accuray Inc., Sunnyvale, CA, USA and Varian, Palo Alto, CA, USA.

FUNDING STATEMENT

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DATA SHARING STATEMENT

The data collected during the study will be stored at secure (de)central research archives at Erasmus MC. Requests for data sharing can be send to the corresponding author. The data will not be publicly available due to privacy and ethical restrictions.

Figure 1. Standard clinical procedures for oropharyngeal cancer patients treated with chemoradiation (CRT) in our center, as well as the study procedures of the COMPLETE trial. The procedures that are specific for the study are an additional tumor biopsy and a liquid biopsy (ctDNA) before treatment. The MR scanning session, including a Intra Voxel Incoherent Motion Diffusion Kurtosis Imaging (IVIM-DKI) diffusion weighted MRI sequence, that is part of the clinical protocol is repeated as part of the study in the second week of treatment, and three months after RT. At the same time points, a second and third liquid biopsy (ctDNA) is acquired.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893-917.

2. Blanchard P, Baujat B, Holostenco V, Bourredjem A, Baey C, Bourhis J, et al. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): a comprehensive analysis by tumour site. Radiother Oncol. 2011;100(1):33-40.

3. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363(1):24-35.

4. Wan Leung S, Lee TF, Chien CY, Chao PJ, Tsai WL, Fang FM. Health-related quality of life in 640 head and neck cancer survivors after radiotherapy using EORTC QLQ-C30 and QLQ-H&N35 questionnaires. BMC cancer. 2011;11:128.

5. Hunter KU, Schipper M, Feng FY, Lyden T, Haxer M, Murdoch-Kinch CA, et al. Toxicities affecting quality of life after chemo-IMRT of oropharyngeal cancer: prospective study of patient-reported, observer-rated, and objective outcomes. Int J Radiat Oncol Biol Phys. 2013;85(4):935-40.

6. Verdonck-de Leeuw IM, Buffart LM, Heymans MW, Rietveld DH, Doornaert P, de Bree R, et al. The course of health-related quality of life in head and neck cancer patients treated with chemoradiation: a prospective cohort study. Radiother Oncol. 2014;110(3):422-8.

7. Van den Bosch L, van der Laan HP, van der Schaaf A, Oosting SF, Halmos GB, Witjes MJH, et al. Patient-Reported Toxicity and Quality-of-Life Profiles in Patients With Head and Neck Cancer Treated With Definitive Radiation Therapy or Chemoradiation. Int J Radiat Oncol Biol Phys. 2021;111(2):456-67.

8. Linge A, Lohaus F, Löck S, Nowak A, Gudziol V, Valentini C, et al. HPV status, cancer stem cell marker expression, hypoxia gene signatures and tumour volume identify good prognosis subgroups in patients with HNSCC after primary radiochemotherapy: A multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). Radiother Oncol. 2016;121(3):364-73.

9. Knegjens JL, Hauptmann M, Pameijer FA, Balm AJ, Hoebers FJ, de Bois JA, et al. Tumor volume as prognostic factor in chemoradiation for advanced head and neck cancer. Head Neck. 2011;33(3):375-82.

10. Lassen P, Lacas B, Pignon JP, Trotti A, Zackrisson B, Zhang Q, et al. Prognostic impact of HPV-associated p16-expression and smoking status on outcomes following radiotherapy for oropharyngeal cancer: The MARCH-HPV project. Radiother Oncol. 2018;126(1):107-15.

11. Rietbergen MM, Brakenhoff RH, Bloemena E, Witte BI, Snijders PJ, Heideman DA, et al. Human papillomavirus detection and comorbidity: critical issues in selection of patients with oropharyngeal cancer for treatment De-escalation trials. Ann Oncol. 2013;24(11):2740-5.

12. Rietbergen MM, Witte BI, Velazquez ER, Snijders PJ, Bloemena E, Speel EJ, et al. Different prognostic models for different patient populations: validation of a new prognostic model for patients with oropharyngeal cancer in Western Europe. Br J Cancer. 2015;112(11):1733-6.

13. van der Schroeff MP, Steyerberg EW, Wieringa MH, Langeveld TP, Molenaar J, Baatenburg de Jong RJ. Prognosis: a variable parameter: dynamic prognostic modeling in head and neck squamous cell carcinoma. Head Neck. 2012;34(1):34-41.

14. Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nat Rev Cancer. 2018;18(5):269-82.

15. Le Bihan D. What can we see with IVIM MRI? Neuroimage. 2019;187:56-67.

16. Lu Y, Jansen JF, Mazaheri Y, Stambuk HE, Koutcher JA, Shukla-Dave A. Extension of the intravoxel incoherent motion model to non-gaussian diffusion in head and neck cancer. J Magn Reson Imaging. 2012;36(5):1088-96.

17. Poot DH, den Dekker AJ, Achten E, Verhoye M, Sijbers J. Optimal experimental design for diffusion kurtosis imaging. IEEE Trans Med Imaging. 2010;29(3):819-29.

18. Sijtsema ND, Petit SF, Poot DHJ, Verduijn GM, van der Lugt A, Hoogeman MS, et al. An optimal acquisition and post-processing pipeline for hybrid IVIM-DKI in head and neck. Magn Reson Med. 2021;85(2):777-89.

19. Naipal KA, Verkaik NS, Sanchez H, van Deurzen CH, den Bakker MA, Hoeijmakers JH, et al. Tumor slice culture system to assess drug response of primary breast cancer. BMC cancer. 2016;16:78.

20. Goldberg SB, Narayan A, Kole AJ, Decker RH, Teysir J, Carriero NJ, et al. Early Assessment of Lung Cancer Immunotherapy Response via Circulating Tumor DNA. Clinical cancer research : an official journal of the American Association for Cancer Research. 2018;24(8):1872-80.

21. Cheng H, Liu C, Jiang J, Luo G, Lu Y, Jin K, et al. Analysis of ctDNA to predict prognosis and monitor treatment responses in metastatic pancreatic cancer patients. Int J Cancer. 2017;140(10):2344-50.

22. van Ginkel JH, Slieker FJB, de Bree R, van Es RJJ, Van Cann EM, Willems SM. Cell-free nucleic acids in body fluids as biomarkers for the prediction and early detection of recurrent head and neck cancer: A systematic review of the literature. Oral Oncol. 2017;75:8-15.

23. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci Transl Med. 2015;7(293):293ra104.

24. Capala ME, Verduijn GM, Petit SF, de Korte MA, Hardillo JA, Sewnaik A, et al. Ex vivo functional assay for predicting radiation treatment response in squamous cell carcinoma of the head and neck. Oral Oncology. 2021;118.

25. Andersson JL, Skare S, Ashburner J. How to correct susceptibility distortions in spin-echo echo-planar images: application to diffusion tensor imaging. Neuroimage. 2003;20(2):870-88.

26. Smith SM, Jenkinson M, Woolrich MW, Beckmann CF, Behrens TE, Johansen-Berg H, et al. Advances in functional and structural MR image analysis and implementation as FSL. Neuroimage. 2004;23 Suppl 1:S208-19.

27. Burgener JM, Zou J, Zhao Z, Zheng Y, Shen SY, Huang SH, et al. Tumor-Naïve Multimodal Profiling of Circulating Tumor DNA in Head and Neck Squamous Cell Carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2021;27(15):4230-44.

28. Lok BH, Setton J, Caria N, Romanyshyn J, Wolden SL, Zelefsky MJ, et al. Intensity-modulated radiation therapy in oropharyngeal carcinoma: effect of tumor volume on clinical outcomes. Int J Radiat Oncol Biol Phys. 2012;82(5):1851-7.

29. Carpén T, Saarilahti K, Haglund C, Markkola A, Tarkkanen J, Hagström J, et al. Tumor volume as a prognostic marker in p16-positive and p16-negative oropharyngeal cancer patients treated with definitive intensity-modulated radiotherapy

Tumorvolumen als prognostischer Marker bei p16-positiven und p16-negativen Patienten mit Oropharyngealkarzinom unter Therapie mit definitiver intensitätsmodulierter Strahlentherapie. Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft [et al]. 2018;194(8):759-70.

30. Studer G, Lütolf UM, El-Bassiouni M, Rousson V, Glanzmann C. Volumetric staging (VS) is superior to TNM and AJCC staging in predicting outcome of head and neck cancer treated with IMRT. Acta Oncol. 2007;46(3):386-94.

31. Vandecaveye V, Dirix P, De Keyzer F, de Beeck KO, Vander Poorten V, Roebben I, et al. Predictive value of diffusion-weighted magnetic resonance imaging during chemoradiotherapy for head and neck squamous cell carcinoma. Eur Radiol. 2010;20(7):1703-14.

32. Lambrecht M, Van Calster B, Vandecaveye V, De Keyzer F, Roebben I, Hermans R, et al. Integrating pretreatment diffusion weighted MRI into a multivariable prognostic model for head and neck squamous cell carcinoma. Radiother Oncol. 2014;110(3):429-34.

33. King AD, Thoeny HC. Functional MRI for the prediction of treatment response in head and neck squamous cell carcinoma: potential and limitations. Cancer Imaging. 2016;16(1):23.

34. Ding Y, Hazle JD, Mohamed AS, Frank SJ, Hobbs BP, Colen RR, et al. Intravoxel incoherent motion imaging kinetics during chemoradiotherapy for human papillomavirus-associated squamous cell carcinoma of the oropharynx: preliminary results from a prospective pilot study. NMR in biomedicine. 2015;28(12):1645-54.

35. Hentschel M, Appold S, Schreiber A, Abolmaali N, Abramyuk A, Dörr W, et al. Early FDG PET at 10 or 20 Gy under chemoradiotherapy is prognostic for locoregional control and overall survival in patients with head and neck cancer. Eur J Nucl Med Mol Imaging. 2011;38(7):1203-11.

36. Kulasinghe A, Perry C, Jovanovic L, Nelson C, Punyadeera C. Circulating tumour cells in metastatic head and neck cancers. Int J Cancer. 2015;136(11):2515-23.

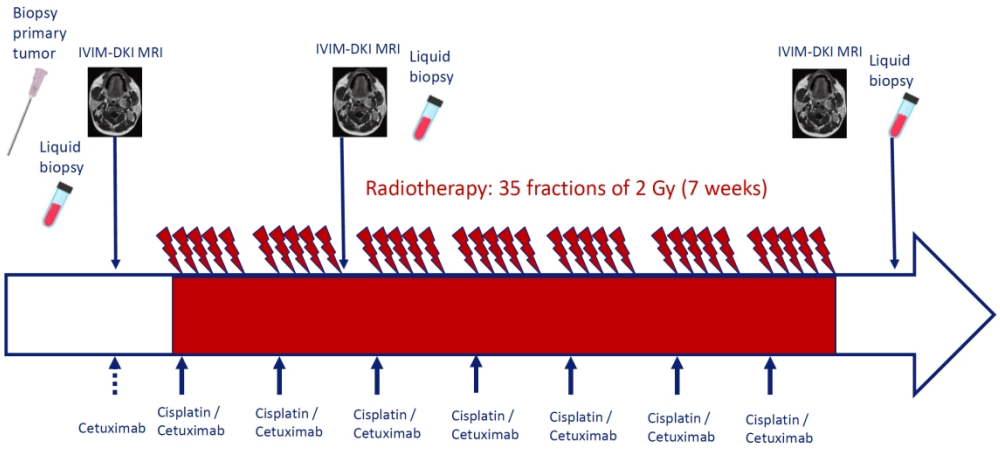

Figure 1. Standard clinical procedures for oropharyngeal cancer patients treated with chemoradiation (CRT) in our center, as well as the study procedures of the COMPLETE trial. The procedures that are specific for the study are an additional tumor biopsy and a liquid biopsy (ctDNA) before treatment. The MR scanning session, including a Intra Voxel Incoherent Motion Diffusion Kurtosis Imaging (IVIM-DKI) diffusion weighted MRI sequence, that is part of the clinical protocol is repeated as part of the study in the second week of treatment, and three months after RT. At the same time points, a second and third liquid biopsy (ctDNA) is acquired.

Appendix 1.

Ancillary study: The BIO-ROC (BIOmarker of treatment Response in Oropharyngeal Cancer) study

All newly diagnosed OPSCC patients in our medical center are asked to participate in the BIO-ROC study that aims to assess the influence of intrinsic tumor properties on the treatment outcomes. This study is a prospective exploratory cohort study for OPSCC patients treated with primary radiotherapy with or without the addition of chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab) with curative intent. The goal is to assess the correlation between tumor *ex vivo* radiosensitivity with clinical response and to build a database of tumor and blood samples for future biomarker identification. For patients with OPSCC accessible during physical examination an additional tumor biopsy will be obtained pretreatment. For patients without histological confirmation of OPSCC and requiring general anesthesia for tumor approach, an extra biopsy next to the diagnostic one will be obtained during a single procedure. For all patients an additional blood sample will be obtained pretreatment, at the end of week 2 during RT, and three months after RT during the clinical response evaluation visit. Clinical outcomes will be assessed within the standard follow-up scheme. In case of tumor recurrence, patients will be approached for obtaining additional tumor and blood samples. Additional informed consent will be asked for the BIO-ROC patients that meet the inclusion criteria of the COMPLETE protocol.

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract **page 2 line 31**
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found **n/a**
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported **page 4 lines 6 - 56, page 5 lines 3 - 5**
Objectives	3	State specific objectives, including any prespecified hypotheses **page 5 lines 8 – 18**
Methods		
Study design	4	Present key elements of study design early in the paper **page 6 line 37**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection **page 6 lines 37 - 46. Page 11 lines 6 - 15**
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up **page 7 lines 50 – 56, page 8 lines 3 – 15 page 9 line 27 page 11 lines 6 - 15**
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed **n/a**
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable **page 11 - 13**
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group **page 11 - 13**
Bias	9	Describe any efforts to address potential sources of bias **n/a**
Study size	10	Explain how the study size was arrived at **page 13 lines 45 - 57**
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why **page 11 lines 27 – 60, page 12 lines 3 - 15**
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding **page 14 lines 8, 38 – 44, page 15 lines 19 - 37**
		(*b*) Describe any methods used to examine subgroups and interactions **n/a**
		(*c*) Explain how missing data were addressed **page 15 lines 37 - 44**
		(*d*) If applicable, explain how loss to follow-up was addressed **n/a**
		(*e*) Describe any sensitivity analyses **page 15 lines 37 - 44**
Results n/a		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed **n/a**
		(b) Give reasons for non-participation at each stage **n/a**
		(c) Consider use of a flow diagram **n/a**
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders **n/a**
		(b) Indicate number of participants with missing data for each variable of interest n/a
		(c) Summarise follow-up time (eg, average and total amount) **n/a**
Outcome data	15*	Report numbers of outcome events or summary measures over time **n/a**
Main results **n/a**	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and

		their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included **n/a**
		(*b*) Report category boundaries when continuous variables were categorized **n/a**
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period **n/a**
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses **n/a**
Discussion		
Key results	18	Summarise key results with reference to study objectives **n/a**
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias **page 17 lines 19 - 21, lines 29 - 38, lines 41 - 59**
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence **n/a**
Generalisability	21	Discuss the generalisability (external validity) of the study results **n/a**
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based **page 19 line 7**

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.