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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 human papilloma virus (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 personalised 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 holistiC early respOnse assessMent for oroPharyngeaL cancer paTiEnts 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 patients with OPSCC.

Methods and analysis This single-centre prospective observational study investigates 60 HPV-negative patients with OPSCC scheduled for primary radiotherapy (RT) 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 3 months after RT), microscopic (with biopsies of the primary tumour acquired before treatment and irradiated ex vivo to assess radiosensitivity), and molecular landscape (with circulating tumour DNA (ctDNA) analysed before, during and 3 months after treatment). The main end point is locoregional control (LRC) 2 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 tumour early during treatment, improves the performance of a predictive model consisting of tumour volume only, for 2 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.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Early tumour response is assessed from macroscopic, microscopic, and molecular perspectives using a combination of novel MRI (Intra Voxel Incoherent Motion Diffusion Kurtosis Imaging), ex vivo radiosensitivity, and circulating tumour DNA 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.

Trial registration number NL8458.

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 HNC. Blanchard et al reported 2 years overall survival (OS) of 50.7% for the chemoradiotherapy 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 3-year locoregional recurrence rate 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 personalised treatment could be investigated, for example, 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 patients with HNC,[8–13] to date no accurate predictive model exists for patients with HPV-negative OPSCC for a number of reasons.[1] Previous studies have focused mainly on patient/clinical characteristics (tumour volume, age, smoking history, comorbidities) in addition to biomarkers of maximum one modality (eg, MRI), while the response of the tumour depends on its entire, complex, multi-layered landscape.[1,42] Many studies focused on pretreatment characteristics only, while a tumour 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 tumours as well as HPV-negative and HPV-positive tumours combined.

The current holistiC early respOnse assessMent for oroPharyngeaL cancer paTiEnts (COMPLETE) study was designed to address these shortcomings directly by (1) studying the entire multilayered tumour landscape based on novel techniques focusing on the macroscopic, microscopic, and molecular landscape; (2) assess changes in the tumour landscape early during treatment and (3) acquire data in a cohort consisting of 60 patients with HPV-negative OPSCC, respectively.

The *macroscopic* tumour 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. 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 IVIM and 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 tumour biopsies will be obtained before treatment as a potential biomarker of clinical outcome. We recently adapted our breast cancer organotypic tumour tissue slice method to be suitable for head and neck tumour tissue (publication in preparation) and developed a protocol for ex vivo radiation treatment of tumour tissue.[19] Using this method, tumour sensitivity to irradiation can be assessed for each individual patient.

Finally, the *molecular* landscape will be studied by analysing liquid biopsies collecting circulating tumour DNA (ctDNA) for molecular tumour 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 DNA fragments derived from tumour cells, which enter the bloodstream after apoptosis or by active shedding of DNA fragments by living tumour cells. Genetic aberrations, such as mutations, can be identified and tracked in ctDNA, and correlated with clinical outcomes. In several tumour 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 tumour 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-centre prospective observational study. In the period of August 2020 until August 2024, 60 patients will be included with histologically proven cT1-2N2-3M0 or cT3-4N0-3M0 HPV-negative OPSCC treated with primary RT and chemotherapy (cisplatin) or epidermal growth factor receptor (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 are 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 tumour early during treatment improves the performance of a predictive model consisting of only tumour volume for the 2 years locoregional control (LRC) after treatment of patients with HPV-negative OPSCC.

Secondary objectives
1. To determine if a relative change in the mean of the diffusion coefficient D in the primary tumour early during treatment improves the performance of a predictive model including tumour volume only for the 3 months response after treatment of patients with HPV-negative OPSCC.
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 potential novel predictive markers for treatment response of patients with HPV-negative OPSCC, 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 patients with HPV-negative OPSCC.

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 RT with chemotherapy (cisplatin) or EGFR-targeted therapy (cetuximab).
► Standard planning MRI (including IVIM-DKI) successfully acquired.
► Included in the BIOmarker of treatment Response in Oropharyngeal Cancer (BIO-ROC) study (see online supplemental 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 5 years.
► Patients with previous irradiation or surgery in a head and neck region overlapping with the current tumour.
► Patients with any physical or mental status that interferes with the informed consent procedure or study procedures.
► Patients with contraindications for MRI (eg, claustrophobia, arterial clips in central nervous system).
► Patients with contraindications for gadolinium contrast (ie, hypersensitivity for gadolinium or an impaired kidney function).

We will continue inclusion until we have 60 evaluable subjects, that is, 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 tumour board and patients will be treated according to the current clinical protocols. Patients will receive 70 Gy intensity modulated radiotherapy or intensity modulated proton beam therapy in 35 fractions combined with cisplatin (100 mg/m^2 body surface area (BSA), once every 3 weeks or 40 mg/m^2 BSA, every once a week) or cetuximab (initial dose of 400 mg/m^2, followed by 250 mg/m^2 weekly, for the duration of RT).

Timing of study procedures

Eligible patients are asked to participate in the BIO-ROC study (see online supplemental 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 immobilised in treatment position (ie, with RT mask). The MRI scan protocol consists of T1-weighted (T1w) Dixon after gadolinium contrast material injection, a T2-weighted (T2w) turbo spin echo, a multi-b-value DWI scan and a DWI
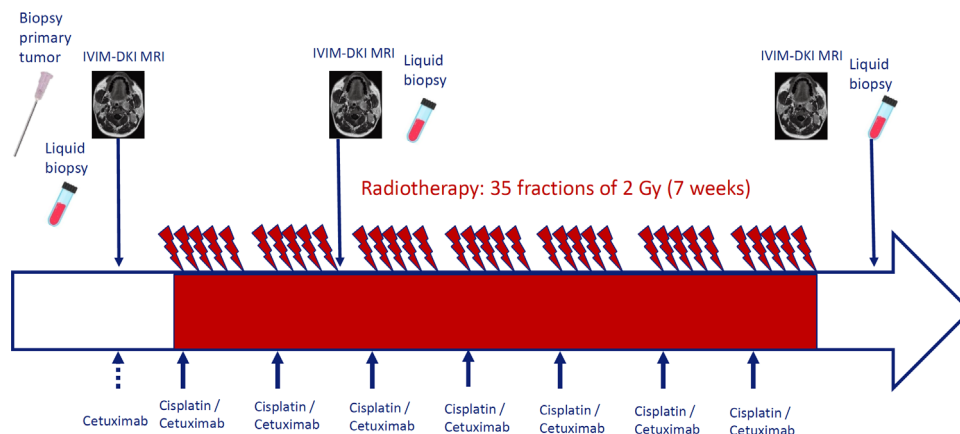

Figure 1 Standard clinical procedures for patients with oropharyngeal squamous cell carcinoma treated with primary radiotherapy with cisplating or cetuximab in our centre, as well as the study procedures of the holistiC early respOnse assessMent for oroPharyngeaL cancer paTiEnts trial. The procedures that are specific for the study are an additional tumour biopsy and a liquid biopsy (circulating tumour DNA (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 3 months after radiotherapy. At the same time points, a second and third liquid biopsy (ctDNA) is acquired.

scan with inverse phase encoding gradient polarity for the purpose of distortion correction (flip angle: 90 degrees; repetition time (TR) : 6700 ms; echo time (TE) 81.8 ms; field of view (FOV) 26×26 cm; 4 mm slice thickness; 0.2 mm gap, 128×128 matrix; bandwith: 1953.12 Hz/pixel). The multi-b-value DWI scan consists of 15 b-values (0, 10, 2×80, 130, 570, 2×770, 2×780, 790 and 4×1500 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 tumour that is accessible during physical examination (with or without histological confirmation), a tumour biopsy will be obtained by a head and neck surgeon during the outpatient clinic visit according to the BIO-ROC study (see online supplemental appendix 1). For patients without histology confirmed OPSCC, and requiring general anaesthesia for proper tumour approach, two biopsies will be obtained during a single procedure, one for the diagnosis and one for the purpose of the study. The tumour biopsies will be sliced into 300 µM thick slices and irradiated ex vivo and cultured for 5 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 tumours.[24] Therefore, all tumour biopsies (of individual patients) used in the current study will be treated with a single dose of 5 Gy. In case more tumour material is available allowing for multiple treatment conditions, separate slices of the same tumour 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 tumour.

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 2 months for the first year following RT. Starting from the second year, the frequency gradually decreases to every 6 months for a minimum of 5 years. LRC at 2 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 tumour 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/mm^2. 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 tumour contours, is projected on top of the scan with b=0 s/mm^2. 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:

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

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 pretreatment 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 with pretreatment is used for the statistical analysis of the primary end point. Next, for $D, f, D^*,$ and K the distribution within the tumour is calculated. From the distribution, a large variety of metrics will be extracted, among others the SD, and the 80th, 90th, 95th, and 99th percentiles, which will be used as input for an exploratory analysis. Moreover, supervoxels will be created to analyse the heterogeneity in the tumour.

The microscopic layer: ex vivo radiation and radiosensitivity testing

The percentage of proliferating cells of the irradiated tumour slices will be compared with untreated tumour slices after 5 days of culture. Proliferation will be detected by 5-ethynyl-2-deoxyuridine (EdU) incorporation and obtained microscopy images will be analysed 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 5 days, using terminale deoxynucleotidyl-transferase dUTP nick-end labeling (TUNEL). 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 analysed as a continuous variable in the exploratory statistical analysis. Change in both parameters compared with the control will be used to describe tumour 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

tumour 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 customised 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 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 to clonal haematopoiesis.

The ctDNA extraction and analysis will be performed on the blood samples acquired pretreatment, acquired in the second week of treatment, and acquired at 3 months post-treatment. The change in the total number of mutant molecules in week 2 compared with 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 2 years (yes/no). Based on relevant literature,[10] within our study population of patients with HPV-negative OPSCC and a smoking history, 37% of the patients are expected to have local tumour progression within 2 years (the primary outcome of interest). We expect to be able to include 60 patients in 4 years, which will lead to approximately 22 events in total. Twenty-two events allow the testing of two explanatory variables based on the rule of thumb that 10 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 LRC at 2 years as dependent variable. According to literature, tumour volume based on the delineated gross tumour volume pre-RT is the most important variable associated with LRC 2 years after treatment among our patient population of only HPV-negative OPSCC patients treated with primary RT 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 2 compared with baseline as determined by the IVIM-DKI scans. The multivariable model including both parameters will be compared with 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 end point at 3 months instead of 2 years, equivalent to the primary objective; the statistical analysis is therefore identical to the one described for the primary end point. The analysis for the first secondary objective will be performed once the 3-month end point is reached for all patients.

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

► Clinical/Patient characteristics such as age, comorbidities, clinical tumour stage.
► IVIM-DKI parameters D, f, $D*$, and K and their distributions within the tumour (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 tumour regions on LRC.
► The established ex vivo radiosensitivity parameters (changes in proliferation and apoptosis on 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 end points will be considered: LRC at 3 months, LRC at 2 years, and OS at 2 years.

Given the large number of variables compared with 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 penalised package in R Statistical software. We will use L1 regularisation 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 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 patients with OPSCC have increased LRC, there is still an urgent need for improvement, especially for patients with HPV-negative OPSCC. 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 tumour 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 multi-layered tumour 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 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 with 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, for example, fluorodeoxyglucose positron emission tomography (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 tumour biopsies to irradiation ex vivo. This novel technique might have profound clinical implications, allowing individualised treatment of patients with OPSCC. However, for several reasons, ex vivo response may not turn out to be representative for patient response. For instance, the biopsy may not represent intratumour heterogeneity of a tumour that may consists of different regions. Furthermore, tumour 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 tumour cells (CTCs), microRNA (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-metastasised) setting, no CTC 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 tumour 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.

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