MIGRHAN - STUDY PROTOCOL

Study Title:	*Microbiota-Guided Radiotherapy for Head and Neck cancer. (MIGRAHN)* *Radioterapia guidata dal Microbiota per Tumori della Testa e del Collo. (MIGRAHN)*
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Background and Rationale

Cancers originating from the head and neck region (head and neck cancer, HNC) constitute a rare and heterogeneous group of neoplasms by anatomical site of origin (oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, cervical esophagus, paranasal sinuses, salivary glands, skin), histology (carcinomas, sarcomas, lymphomas) and biological behavior (different degrees of local aggressiveness and tendency to develop distant metastases). The majority of these tumors (90%) originate from the squamous epithelium lining the mucosa and are called "Carcinomas."

The choice of therapeutic strategy in individual cases is determined by multiple factors: the extent of the primary tumor, the presence or absence of lymph node disease, the presence of patient comorbidities. In any case, the choice of treatment must always be multidisciplinary and individualized, taking into account the patient's need for preservation of life, function and organ, and the preferences expressed by the patient, as well as the best oncological outcome regarding phonation and swallowing. Surgery (CT), radiotherapy (RT) and chemotherapy have been used for years in various combinations in the treatment of head and neck tumors. To achieve the best oncological result, a single therapeutic modality can be chosen for limited-stage tumors (surgery or radiotherapy), while the combination of multiple therapeutic strategies is indicated for advanced-stage tumors.

Radiotherapy is the discipline that uses radiation (Gamma or X rays) or heavy particles (Hadrons: Protons or Carbon Ions) for therapeutic purposes, exploiting their properties to act on cellular DNA to destroy neoplasms. Approximately 75% of patients with squamous cell carcinoma of the cervical-cephalic region benefit from radiotherapy treatment during their treatment course. Therapeutic outcomes for head and neck neoplasms remain unsatisfactory and heterogeneous, with 5-year survival rates varying between 28% and 67% overall, 80-90% for early-stage tumors and 40% for locally advanced head and neck tumors.

Furthermore, patients with HNC experience side effects that often negatively affect quality of life during and after treatment. Oral mucositis –a painful inflammation and ulceration of the oral lining caused by radiation or cytotoxic agents– is considered the most serious and debilitating complication in HNC treatment. Oral mucositis develops on average in 70% of patients with HNC, making it difficult for them to eat, drink and speak. It can lead to pain, superinfections and malnutrition, and can be a limiting factor for RT dose escalation.

Therefore, the development of strategies to reduce RT-induced side effects and improve therapeutic outcomes for patients with HNC is essential. Despite a vast amount of clinical-genomic data on tumors available, no relevant biomarkers for personalized treatments have been identified and are currently used in the clinical practice.

The human microbiota comprises the community of all organisms living within or on the surface of our body, including bacteria, archaea, fungi and viruses, and has demonstrated great potential in recent years as a determinant of therapeutic response to cancer treatments. Numerous studies indicate that intestinal microorganisms play an active role in modulating the effects of cytotoxic agents and immune checkpoint inhibitors used in immunotherapy.

Several observational studies have shown the existence of statistical associations between the oral microbiota and various factors related to HNC and its therapeutic course, including correlations between changes in bacterial genus composition and oral mucositis progression and worsening, and between the relative abundance of specific species profiled from patient saliva and cancer onset and progression. However, these studies rely on 16S rRNA metagenomic sequencing technology, which has several known limitations: 16S rRNA sequencing can reliably characterize the bacterial component of the oral microbiota up to the taxonomic level of genera and, more importantly, does not provide direct information about metagenome function. This has limited the ability to identify or discover specific bacterial strains or molecules associated

with HNC treatment outcomes, and has prevented obtaining a broader understanding of the role that archaea, fungi and viruses present in saliva may play in HNC.

Evidence from several experimental studies based on preclinical models also supports the hypothesis that the oral microbiota influences cancer treatment. However, to date, a direct causal association between the human oral microbiota and radiotherapy modulation has not been established through analysis of observational data from HNC patients.

The research question that the study intends to address is to fill the knowledge gap in our understanding of the role that the microbiota and potential microbiota-targeted therapies, such as the use of antibiotics, could play in HNC treatment. The study hypothesis is that it is possible to establish causal relationships between functional traits of the human oral microbiota and the effectiveness of radiotherapy in head and neck carcinoma treatment using the statistical framework of causal inference. The identification of such causal knowledge is essential for the design and implementation of microbiota-guided radiotherapy, in which the patient's oral microbiota is profiled before treatment initiation and evaluated against the risk of developing oral mucositis and/or an unfavorable outcome.

Evaluating, from a microbiota-based perspective, whether different RT plans can lead to tolerable or intolerable side effects would be of fundamental importance for designing personalized radiotherapy programs that could increase tumor control. Furthermore, since the project involves microbiota analysis at the functional metagenomic level, it will be possible to extrapolate from the inferred relationships the potential mechanisms through which the microbiota modulates RT and exploit them to design new clinical strategies involving microbiota manipulation. This may include the administration of specific dietary supplements, antibiotics or probiotics to predispose the patient to RT with a consequent reduction in the risk of developing side effects and improving treatment efficacy and outcomes.

Study Objectives

To test the hypothesis, the innovative idea of the study is to use the causal inference framework to model compositional change in the microbiota during HNC treatment as the effect of an intervention concomitant with RT. To implement this framework, it will be necessary to generate and analyze metagenomic data that will advance the state of the art of our knowledge about the oral microbiome in cancer. The study will pursue the following objectives:

• **Objective 1:** Creation of an oral microbiota dataset in HNC patients, including bacteriome and virome components using shotgun metagenomic sequencing, with data associated with treatment outcomes and side effects.

• **Objective 2:** Estimation of the causal effect of oral microbiota functional traits (from combined virome and bacteriome components) on radiotherapy efficacy and toxicity in HNC.

• **Objective 3:** Development of microbiome-based predictive models for tumor reduction, recurrence and acute oral mucositis development in patients with head and neck tumors.

To achieve these objectives, clinical data and salivary samples will be collected from n=96 patients with head and neck cancer enrolled over a 3-year period, which will be combined with data from the MicroLearner study (2017-2019) available at the IRCCS National Cancer Institute Foundation of Milan (ethics committee approval INT11-17 of 23/01/2017) bringing the final dataset to a total of n=276 patients.

Study Design and Setting

MIGRAHN is a single-institution observational cohort study with prospective follow-up lasting approximately 6 years. The study will enroll at least n=96 consecutive patients with head and neck tumors who are candidates for curative radiotherapy treatment over a 36-month (3-year) time frame.

The treatment, toxicity monitoring and follow-up (3 years) of these patients will be in accordance with routine clinical practice based on national and international guidelines. Enrollment will occur exclusively at the IRCCS National Cancer Institute Foundation of Milan with enrollment start date scheduled for June 2025.

Study Schedule

	Baseline (from -28 to 0 days)	Radiotherapy	End of Treatment	Follow-up
Informed consent	X			
Demographic data	X			
Medical history	X			
Dental evaluation	X			X
Clinical evaluation	X			
Questionnaires	X	X	X	X
Toxicity	X	X	X	X
Blood chemistry tests	X	X	X	X
Urine examination	X			
Thyroid function test	X			X
Coagulation test	X			
Salivary sample (microbiota)	X	X	X	
Salivary sample (metabolome)	X	X		
Dosimetric data (DICOM-RT)			X	
CT scan/mp-MRI	X	X		X
Whole body FDG-PET/CT scan or bone scan	X			X
Microcirculation evaluation	X	X		
Survival data			X	X

Pre-Radiotherapy Treatment Evaluation

Patients enrolled in the study will undergo a pre-treatment evaluation including:

1. Registration of demographic data;
2. Collection of oncological history, including tumor stage, HPV status determination for oropharyngeal tumors, lifestyle information (smoking and alcohol consumption); collection of non-oncological history with registration of comorbidities;
3. Clinical evaluation including ENT examinations with fiberendoscopy, weight recording and performance status (PS) according to ECOG;
4. Baseline multiparametric MRI (mp-MRI) of the facial complex and neck with and without contrast medium (preferably to be performed no later than 4 weeks from RT initiation);
5. 18-fluorodeoxyglucose-PET or chest-abdomen CT scan, according to stage and histology, for the search for distant metastases;
6. Routine blood tests including complete blood count, liver function, kidney function, C-reactive protein, neutrophil and lymphocyte count and TSH;
7. Dental evaluation and possible dental restoration (within two weeks of treatment initiation)
8. Registration of baseline locoregional toxicities according to CTCAE v. 5.0;
9. Assessment of global quality of life, lifestyle and nutrition, xerostomia reported by the patient through the administration of validated questionnaires: QLQ-C30, QoL HN35, XQ for xerostomia, lifestyle and nutrition questionnaire;
10. Collection of a saliva sample in "OMNIgene SALIVA DNA and RNA kit (OMR-610)" for pre-radiotherapy salivary microbiota profiling;
11. Collection of a saliva sample in 50 mL Falcon tubes for pre-radiotherapy salivary metabolome profiling as complementary data to the microbiota.
12. Non-invasive microcirculation assessment using the GlycoCheck® system.

CT simulation procedures (with or without contrast medium) will be initiated no later than 2 weeks before the start of radiation treatment. Intensity-modulated radiotherapy treatment using photons X≥6 MV and daily setup control using IGRT techniques according to institutional standards is planned. Radiotherapy will be delivered with conventional or moderately accelerated hypofractionated fractionation (≤2.2 Gy/fraction) with total prescription doses of ≤70 Gy (2 Gy-equivalents) in the definitive setting or 54-66 Gy (2 Gy-equivalents) in the postoperative setting. The choice of irradiation targets and dose constraints for organs at risk (OAR) will be made according to institutional guidelines. OARs will be defined in accordance with recent international guidelines [Brouwer CL, Radiother Oncol, 2015].

Evaluation During Radiotherapy Treatment

Evaluation during treatment will include:

1. Once a week during radiotherapy: registration of district toxicity by healthcare operator (CTCAE v 5.0), registration of PS according to ECOG;

2. Second week from radiotherapy start: only on patients receiving radical treatment mp-MRI of facial complex and neck without contrast medium;
3. End of last week of radiotherapy: administration of QLQ-C30, QoL HN35, XQ questionnaires for xerostomia.
4. Second week from radiotherapy start and end of last week of radiotherapy: collection of a saliva sample in "OMNIgene SALIVA DNA and RNA kit (OMR-610)" for salivary microbiota profiling at two weeks from radiotherapy start and post-radiotherapy;
5. Second week from radiotherapy start: collection of a saliva sample in 50 mL Falcon tubes for salivary metabolome profiling at two weeks from radiotherapy start as complementary data to the microbiota;
6. End of last week of radiotherapy: collection of dosimetric data (DICOM-RT);
7. Blood chemistry tests during treatment will be prescribed according to usual clinical practice.
8. Second week from radiotherapy start: non-invasive microcirculation assessment using the GlycoCheck® system.

Follow-up

At 3, 6, 12, 24, 30, 36 months after radiotherapy completion, patients will be evaluated with:

1. ENT clinical evaluation including fiberendoscopy, body weight recording and PS according to ECOG.
2. Toxicity registration by healthcare operator according to CTCAE v 5.0.
3. QOL questionnaires completed by patient QLQ-C30, QoL HN35, XQ.
4. Survival data.

At 3, 6 and 12 months after radiotherapy completion mp-MRI of facial complex and neck with and without contrast medium (examination aimed at evaluating changes in healthy tissues involved in irradiation), and subsequently within oncological follow-up controls according to internal guidelines.

At 12, 24 and 36 months a dental evaluation is planned.

Study Population

Inclusion Criteria

Patients may be enrolled in the study with:

1. Age ≥ 18
2. ECOG Performance Status ≤ 3
3. Histological diagnosis of squamous cell, undifferentiated, epithelial glandular and non-glandular carcinoma (adenoid cystic carcinoma, adenocarcinoma, mucoepidermoid carcinoma, neuroendocrine

carcinoma, etc.) originating from oral cavity, oropharynx, nasopharynx, hypopharynx larynx, salivary glands, paranasal sinuses, unknown primary.

4. Stage III-IV non-metastatic for pharyngeal, laryngeal and unknown primary tumors according to AJCC 7th edition. Patients with stage III-IV originating from salivary glands or paranasal sinuses and patients in stage I-II pharyngeal-laryngeal site will be included only if prophylactic irradiation of neck lymph node stations is planned and/or when the volume of oral and oropharyngeal mucosa as well as the volume of swallowing-related structures is included in the irradiated volume.

5. Indication for treatment in definitive or adjuvant setting and in association or not with systemic therapy (concomitant preceded or not by neoadjuvant chemotherapy. Adjuvant systemic treatment will be admitted for particular advanced stages of pharyngeal carcinoma according to internal guidelines).

6. Formal acceptance of study participation modalities (written informed consent).

Exclusion Criteria

Patients will be excluded from the study with:

1. Previous radiotherapy in the head-neck region.

2. Carriers of connective tissue diseases (i.e., lupus erythematosus or scleroderma) or other synchronous ENT tumors except superficial skin tumors and surgically treated carcinomas in situ for which radiotherapy or systemic treatment is not planned.

3. Lack of formal acceptance of study participation modalities (written informed consent).

4. Indication for treatment in postoperative setting.

Study Outcomes

Primary Endpoint

• **Local control.**

The primary endpoint is local tumor control at 12 months of follow-up taking into account partial and complete responses according to RECIST 1.1 radiological criteria used in oncology. Tumor volume analysis from MRI images between pre-RT and 3 months post-RT will be used to quantify tumor volume reduction (shrinkage) in patients undergoing exclusive curative RT. Tumor volume analysis from MRI images between 3 months post-RT, and 6 and 12 months post-RT will be used to quantify tumor volume recurrence.

Secondary Endpoints Related to Cancer Treatment

• **Early response.**

Tumor volume analysis from MRI images between pre-RT and 2 weeks from radiotherapy start will be used to quantify early treatment response in patients receiving radical treatment.

• **Progression-free survival (PFS, at 3 years of follow-up).**

PFS will be defined as the time interval from treatment start to the occurrence of the first of two events between disease progression and death from any cause.

• **Disease-free survival (DFS, at 3 years of follow-up).**

DFS will be defined as the time interval from treatment start to the occurrence of the first of two events between the appearance of metastases or a new disease relapse and death from any cause.

• **Overall survival (OS, 3 years, at 3 years of follow-up).**

OS will be calculated as the time interval from treatment start to death from any cause. Observation times of patients who do not have events at the time of results analysis will be censored at the date of their last information on living status.

Secondary Endpoints Related to Radiation-Induced Toxicity

• **Acute toxicity;**

• **Late toxicity (at 3 years of follow-up).**

Toxicity evaluation will be performed by the radiation oncologist according to CTCAE v. 5.0 recommendations, through questionnaire completion. CRO (clinician reported outcome) collected weekly during RT will be used to define longitudinal descriptors of treatment side effect development and severity. Descriptors of interest will include average mucositis grade, G3+ mucositis grade and maximum mucositis grade. Beyond CRO-based descriptors, the possibility of defining quantitative toxicity descriptors from normal tissue analysis in MRI images through textural feature extraction will be evaluated.

Sample Size

The study size (n=96 patients) combined with available MicroLearner study patient data (n=180 patients) is designed to provide a sufficient number of data points (n=276 patients) to estimate the causal effect of the microbiota on the primary endpoint (Objective 1) and to develop personalized predictive models of treatment efficacy and side effects based on clinical, imaging, dosimetric and pre-radiotherapy microbiota data (Objective 3). Machine learning techniques rather than statistical tests will be used for statistical analyses and, therefore, it is not possible to know a priori the minimum number of data points required to optimally learn predictive models and counterfactual outcome approximation functions from the data.

Based on preliminary clustering analyses on microbiota composition variation in a population of n=159 HNC patients, three clusters of sizes n=45, n=51 and n=63 patients were identified, showing G3+ acute oral mucositis (AOM) rates of 40%, 24% and 35%, respectively. Analysis of 1-year progression-free survival (PFS) revealed that cluster 1 patients (n=30) have an unfavorable prognosis (HR=3.43, P=0.019 log-rank test) compared to cluster 3 patients (n=43). According to these results, we can assume that there exists a characteristic microbiota change associated with RT side effect reduction, observed in 32% of patients (cluster 2, 51/159). In this patient subgroup (n1, corresponding to cluster 2), the observed G3+ AOM rate is 24%, while in the remaining 68% of patients (n2, rest of population corresponding to union of clusters 1 and 3), the observed G3+ AOM rate is 59%. A two-group Wald test with population groups n1, n2 with ratio n2:n1=2 presenting different G3+ AOM incidence (24% vs 59%) and with significance level equal to 0.05 (two-tailed) and power equal to 0.8 returns minimum sample sizes to detect differences associated with mucositis equal to n=n1+n2=69 with n1=23 and n2=46. The final study size combined with MicroLearner

patient data (n=276 patients) will be at least 3.5 times larger than the estimated sizes to appreciate differences in acute toxicity incidence and treatment efficacy in microbiota clusters.

Statistical Analysis Plan

Sequencing Data Analysis

Demultiplexed sequences will be processed using standard bioinformatics pipelines for taxonomy assignment and functional description of the sample metagenome (e.g., MetaPhlAn, Kraken). The sparsity and compositional nature of microbiome data will be accounted for using zero imputation through Geometric Bayesian Multiplicative Replacement methods and log-ratio transformation. For microbiota variation analysis, both bacterial species and functional characteristics based on gene mapping from metagenomes to metabolic pathways and other types of functional annotations using well-established databases such as KEGG and REACTOME will be considered as fundamental units.

Imaging Data Analysis

Image analysis for tumor characterization and evaluation of tumor control and radiation-induced effects on normal tissues will be performed on multiparametric magnetic resonance (mp-MR) images, including 3D sequences of T1-w, T2-w, DWI and T1-contrast images acquired before treatment start and during follow-up. Tumor volume segmentation on mp-MR images and normal structure segmentation will be performed using open-source auto-segmentation algorithms (e.g., 3D Slicer). Tumor volume variation quantification will be performed by measuring differences in segmented image regions.

Causal Inference

For each HNC patient in the final generated dataset, we will have associated data to define the following variables: patient covariates such as age, cancer stage, smoking status (X), salivary microbiome data profiled before (Mo) and during RT (M1), and various treatment outcomes of interest (Y). The innovative idea will be to model the change in microbiota composition (DM) during RT as the effect of an intervention concurrent to RT. The causal inference framework developed by Rubin and Neyman will be applied based on the notion of potential outcomes Y(T=k) where k is an index that identifies different treatment levels. The causal effect is defined as the comparison of potential outcomes, for the same patient at the same time point after treatment. The fundamental problem of causal inference is that, for each patient, at most one of the potential outcomes can be realized and observed in the data. To estimate the causal effect of the microbiota on RT, the generated data (n=276) will be used to predict unobserved outcomes (counterfactual outcomes) through a machine learning-based covariate adjustment procedure.

Covariate Adjustment and Predictive Model Development

We will consider different functional forms to learn from data the algorithm that best predicts observed outcomes Y and best simulates counterfactual outcomes. We will train linear regression models and non-linear models such as random forests and neural networks using machine learning routines available in R or Python. To evaluate model performance, we will implement k-fold cross-validation and measure mean squared error (RMSE) or quantify the discrepancy between predicted and actual classes. We will evaluate the significance of the estimated average microbiota effect (AME) by performing hypothesis testing using a t-test on the t-statistic of AME/SE[AME], as well as non-parametric approaches based on permutations or bootstrap.

Administrative Aspects

Study Funding

This observational study is part of the research project funded by the MFAG-2024 call n. 31114 with principal investigator Dr. Jacopo Iacovacci and the related costs are included in the project's economic-financial budget.

Ethical Considerations

All investigators declare that this study protocol is in compliance with ethical principles and good clinical practice. They also declare their commitment to comply with the deontological rules for statistical or scientific research treatments published pursuant to art. 20 c. 4 of Legislative Decree 10 August 2018, n. 101 -- Annex A.5 to Legislative Decree 196/2003. They finally declare that the opinion of the ethics committee CET LOMBARDIA4 has been requested and communication sent to the competent authority.

Informed Consent Acquisition and Data Processing

The informed consent acquisition procedure will include two integrated phases:

1. Explanatory interview with a study investigator, during which purposes, risks/benefits, withdrawal rights and pseudonymization methods are illustrated.

2. Signing of the form in paper or electronic format (with qualified digital signature).

Verification procedures will include:

- Systematic registration of refusals and motivations (dedicated register).
- Quarterly audit on exclusion cases due to lack of consent.

The process will respect the requirements of art. 110 of Legislative Decree 196/2003 and the AIFA Guidelines 2022, ensuring compliance with the Deontological Regulation for statistical research (Art.3 c.3).

Conflict of Interest

None of the investigators participating in the study have financial interests to declare in relation to the entirety of the study or each of its parts.

Responsibilities and Publication Policies

Role of Sponsor and Investigators

The sponsor had no role in the study design. All investigators in the study participated in the study design and will contribute to data collection, management, analysis and interpretation, report writing. All investigators will appear as authors in any scientific publications based on the results of this study.

Data Ownership

The ownership of data and samples generated for the study will belong to the sponsor (IRCCS National Cancer Institute Foundation of Milan).

Publication Policies

The study results will be made available to the public through conference presentations and publications in scientific journals.