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Title

IMMUNE SYSTEM MODULATION AND OUTCOME IN HIGH-RISK CUTANEOUS SQUAMOUS CELL CARCINOMA TREATED WITH SURGERY AND RADIOTHERAPY: A PROSPECTIVE STUDY.

Acronym: HR-cSCC

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PROTOCOL

1. BACKGROUND INFORMATIONS AND RATIONALE

1.1. CUTANEOUS SQUAMOUS CELL CARCINOMA

Cutaneous squamous cell carcinoma (cSCC) is the second most common form of skin cancer and derives from keratinocytes. It accounts for 20% of skin cancers but recently studies show a rising of incidence, therefore reducing the difference with basal cell carcinoma (BCC). cSCC is more frequent in Caucasian population and the most frequent site is head and neck. The most significant risk factors are age (it is more common from 60 years), fair skin, hair and eyes, sun exposure or indoor tanning. A very important risk factor is immunosuppression, particularly in patients who received a solid organ transplant, with a history of hematological malignancies or in patients with chronic immunosuppressive therapy because of autoimmune diseases. In these cases, the risk is from 5 to 100-fold higher. Usually, cSCC is diagnosed at an early stage as localized disease, more rarely it is diagnosed as locally advanced or metastatic at diagnosis. For local cSCC the goal is a radical surgery that could be a classical surgical excision or a Mohs Micrographic Surgery. In any case, for radical intent, margins must be negative. In particular, margins should be from 4 mm wide to 10 mm according to the risk of primary lesion. So far, there is no consensus about sentinel lymphnode role, due to the small number of patients enrolled in clinical studies aimed at evaluating this therapeutic strategy. After histopathological confirmation of cSCC, an important step is to establish the risk of recurrence. cSCC patients need to be distinguished as having low or high risk of recurrence on the basis of several clinical and histopathological risk factors. In fact, in case of high risk cSCC further adjuvant treatment should be evaluated in multidisciplinary discussion.

The following factors should lead to consider the employment of adjuvant radiotherapy in the setting of operated cSCC:

- close or positive margins that cannot be corrected with further surgery (secondary to morbidity or adverse cosmetic outcome)
- gross perineural spread
- setting of recurrence after a prior margin-negative resection
- stage T3 and T4
- desmoplastic or infiltrative tumors in the setting of chronic immunosuppression
- at pathological exam, cSCC involving regional lymphnodes, with the exception of a single, small (<3 cm) cervical lymph node harboring carcinoma, without extracapsular extension.

In terms of cSCC staging there are two major classification systems: the AJCC (American Joint Commettee on Cancer) 8th edition that considered T stage and N stage and the BWH (Brigham and Women's Hospital) that considers only T stage.

An alternative to surgery for inoperable or difficult to operate cSCC is primary radiotherapy (RT). Another application of RT is the adjuvant setting. It is used as clinical practice for operated cSCC with positive margins where there is not space for re-surgery or in case of high risk cSCC, in particular showing extensive perineural or large nerve involvement, or in patients with positive nodes (particularly with extracapsular extension). Unfortunately, the value of adjuvant RT is still debated due to the lack of randomized prospective trials. The application of immunotherapy with anti-PD1 agents in adjuvant setting is a new investigation field. Trials are ongoing to evaluate the efficacy of immune-agents in high risk cSCC after RT.

For locally advanced, recurrent and metastatic cSCC, systemic treatments could be used. An important study field is the application of immunotherapy in this kind of patients. The rational behind treating cSCC with anti-PD1 agents is based on different reasons. Primarily, cSCC are radiation induced tumors so they exhibit a high tumor mutational burden (TMB) that may act as neo-antigen in stimulating cytotoxic T cells. Secondarily, the disease is strongly associated with immunosuppression. Several studies with anti-PD1 were conducted till cemiplimab was approved in first line setting for locally advanced and metastatic cSCC showing a huge, rapid and durable response (ORR about 50%) with an acceptable rate of adverse events. Pembrolizumab is another immune checkpoint inhibitor employed in cSCC.

Considering other systemic treatments, epidermal growth factor receptor inhibitors were tested. Cetuximab is an intravenous EGFRi showing responses in locally advanced or recurrent/metastatic head and neck cancers in combination with RT or chemotherapy, respectively. Also, classic chemotherapy regimen based on cisplatin, carboplatin, 5-FU and taxanes in monotherapy or in combination therapy were administered in advanced setting. Efficacy data were derived from small case series and a limited number of randomized trials. With these systemic agents, duration of response is limited and toxicity is often an issue when treating elderly patients with multiple comorbidities (1-2-3-4-5).

1.2. CLINICAL APPLICATION OF ADJUVANT RADIOTHERAPY IN cSCC

The value of adjuvant RT is still debated due to the lack of randomized prospective trials, however, there is general consensus on its added value after surgery for high-risk cSCC. As demonstrated in the phase 3 randomized TROG 05.01 trial, the combination of surgery and adjuvant RT results in a sustained rate of loco-regional control (88% and 82% in the control arm of the study at 2 and 5 years, respectively) (6). Large retrospective data from tertiary centers also point towards a survival benefit associated with RT receipt, particularly for those patients with perineural invasion and regional disease (7). Overall, there are some condition defined at high risk in which radiotherapy is performed as post-operative treatment. The principal risk factors that should be considered for post-operative

radiation treatment are the presence of positive margin after surgery, nodal involvement, perineural invasion and lympho-vascular invasion. Other prognostic factors are site of disease, poorly differentiation, type of histology and recurrence.

Usually, post-operative radiation therapy consists of a minimum biologically Equivalent Dose of 2 Gy per fraction to the site of primary disease for a total dose of 50-54 or 60-66 Gy depending on the margin status.

The Head and Neck Cancer International Group (HNCIG) Consensus Guidelines (8) should be regarded as the reference paper for the delivery of postoperative RT in high-risk cutaneous squamous cell carcinoma of the head and neck. Briefly, a high-risk tumor volume for the primary tumor (CTVp_HR) and nodal disease (CTVn_HR) should be recognized in the delineation phase based on all clinical, radiological and pathological findings, enlarged of a 5-mm margin (edited for anatomical barriers) and be targeted to a total dose of 60 Gy at conventional fractionation, whereas those areas deemed at substantial risk of microscopic disease such as positive surgical margin or pathologic lymphnodes with extranodal extension may warrant a boost dose up to 66 Gy (CTVp_HR_Boost or CTVn_HR_Boost). All those areas deemed at low risk of harboring microscopic disease should be encompassed within the low-risk tumor volume, both for the primary and nodal disease (CTVp_LR and CTn_LR, respectively). Simulation, treatment planning, treatment delivery and verification should be performed according to standard practice. Intensity-modulated radiation therapy (IMRT) should be regarded as the minimum technical requirement. As suggested by the HNCIG consensus guidelines, when IMRT is used, a common treatment schedule employing a simultaneous-integrated boost (SIB) technique may consist of a 30-fraction course whereby 63 Gy, 60 Gy and 56 Gy are delivered to the PTV_boost, PTV_HR and PTV_LR, respectively.

Radiation therapy can cause several side effects such as rashes, dry, itchy and frail skin, photosensivity, oedema, fatigue. For head and neck treatment frequent adverse events are also sore throat, dry mouth, cough and difficult swallowing (9).

1.3. IMMUNE SYSTEM IN cSCC AND IMMUNO-MODULATING EFFECTS OF RADIOTHERAPY

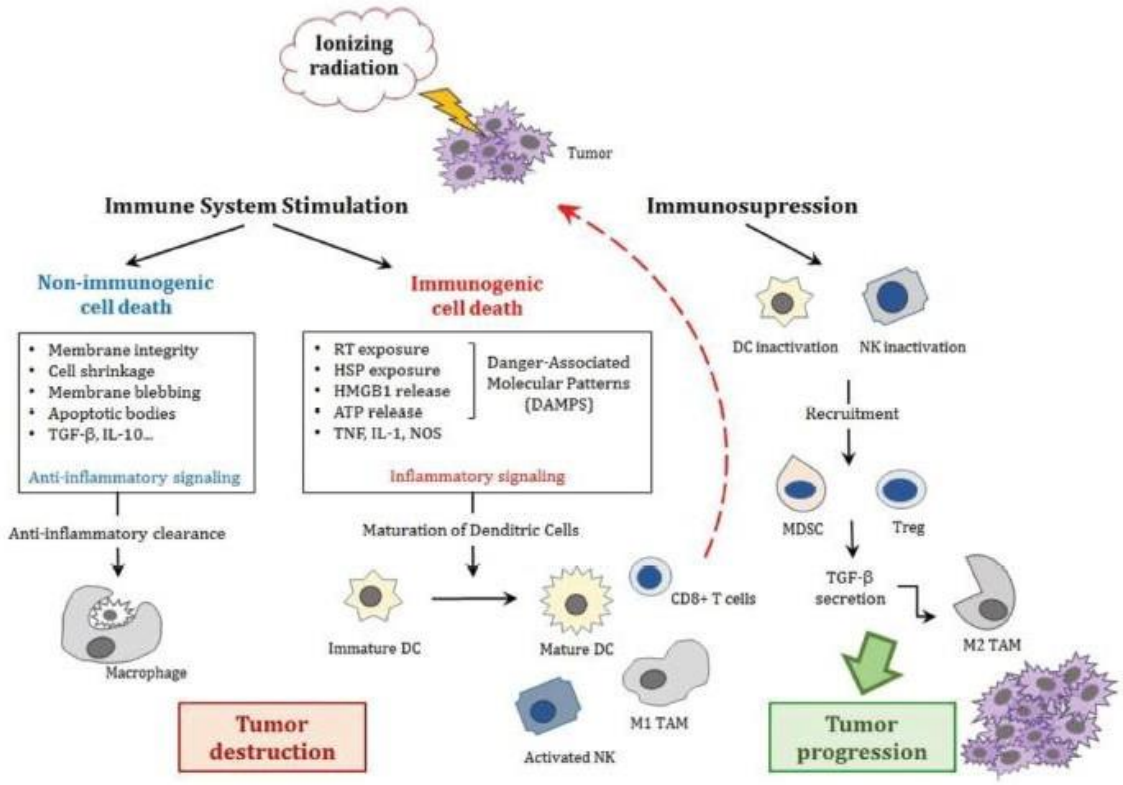
cSCC is a neoplasm strictly related to immunosuppression: in this context, cSCC could be multiple, recurrent and more aggressive. This statement highlights the importance to study the relationship between this tumor and circulating immune-cell and tumor microenvironment (TME). The immune mechanisms involved in tumors development are complex and related to the interplay of immunosurveillance/immunoediting and the TME that is characterized by dynamic intercellular communications via networks of chemokines, cytokines, growth factors and inflammatory remodeling enzymes. A lot of immune-circulating cells are involved in tumor growth control and in cSCC development, as neutrophil, T lymphocytes (CD8 and CD4, natural killer cells), B lymphocytes, dendritic cells, myeloid-derived suppressor cells, chemokines and cytokines (IL-6, IL-10, IL-2, IFN-γ, TGF-β) (10). This equilibrium between TME and circulating immune cells plays an important role

in patient's outcome. Moreover, oncologic treatments as chemotherapy and radiotherapy modifies and influences patient's immune system. In particular, several retrospective studies demonstrated the alterations induced by radiotherapy in different cancer subtypes and their effects on outcome. In patients affected from pancreatic, head and neck, non-small cell lung cancer and cervical cancer, radiation or chemoradiation treatments induced lymphopenia (defined as lymphocytes < 500) and the occurrence of elevated neutrophil- to- lymphocytes ratio in many patients who were characterized from worse prognosis in terms of reduced progression or disease-free survival and overall survival. Furthermore, worse outcomes were related to dose, volume and site of irradiation. For non-small cell lung cancer a connection between radiation-induced lymphopenia and Gal-1 secretion was found. Even if there are not in literature studies evaluating the relation between lymphopenia and cSCC, we can hypotheses that the same scenario could interest patients affected from neoplasm as cSCC that is also particularly dependent from patient's immune system. (11-12).

The relation between immune system and neoplasm has become an important field of research with immunotherapy development in these last few years. Radiotherapy causes modifications on circulating immune cells and TME. In fact, irradiation provokes endothelial cells damages and inflammation causing inhibition of CD8+ T lymphocytes infiltration into tumors and immunosuppressive pathways activation. This immunosuppressive effect is represented by accumulation of radioresistant suppressor cells as macrophages, myeloid-derived suppressors cells, and regulatory T cells. But, on the other hand, irradiation can also activate the immune response through release and presentation of antigens, priming and activation of T lymphocytes, recruitment and accumulation of T cells in the tumor, enhancing inflammation and increasing IFN-γ expression (13-14). So, radiotherapy effect on immune system and TME is very complex and two-fold.

We lack studies evaluating the interplay between immune system and cSCC before and after radiation treatment, investigating the effect on TME. The aim of this study is to evaluate the effect of radiotherapy-induced lymphopenia in cSCC on outcomes and radiotherapy modifications in terms of changes in the profile of circulating immune cells.

Fig. 1 The two-fold effect of radiotherapy on cancer cells and TME



1.4 MOLECULAR ASPECTS OF cSCC

The most significant risk factors involved in cSCC pathogenesis are sun exposure, UV rays and indoor tanning. These elements are associated with elevated tumor mutational burden (TMB). It is well known that cSCC carries more mutation than other common cancer (5 times the mutation rates in lung cancer and 4 times in melanoma) but, nowadays, there are not validated evaluations and classifications that takes into account a molecular profile of cSCC. Targeting TMB in cSCC could be an instrument to classify these tumors in different levels of risk of recurrence and/or metastatic spread. The cSCC risk profile is only based on clinic-pathological risk factors listed in NCCN Guidelines. The integration between clinical-pathological risk factors and molecular or immunological tumor profiles may improve the prognostic ability and consequently may refine management of localized lesions. This could prompt to tailor adjuvant treatments and define personalized follow up programs.

A 40-gene expression signature test has been recently developed and validated. It classifies patients with cSCC into three groups: low risk of recurrence, high risk and highest risk, for this latter category the positive predictive value is 60%. Many of the discriminant genes comprises in the test have been previously reported in cSCC and/or have known function in cancer pathways.

The application of this test could be another step to better classify cSCC and to choose a more adequate post-operative treatment based on molecular risk of recurrence. (15-16-17)

Fig. 2 Some of discriminant genes included in the 40 gene expression panel.

GENE ID	GENE NAME	SOURCE
ACSBG1	Long-chain-fatty-acid--CoA ligase ACSBG1	1
ALOX12	Arachidonate 12-Lipoxygenase, 12S Type	1
APOBEC3G	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3G	1
ATP6V0E2	ATPase H+ Transporting V0 Subunit E2	1
BBC3	Bcl-2-binding component 3	2
BHLHB9	Basic Helix-Loop-Helix Family Member B9	8
CEP76	Centrosomal protein of 76 kDa	1
DUXAP9	Double Homeobox A Pseudogene 9	8
GTPBP2	GTP Binding Protein 2	8
HDDC3	Guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase MESH1	8
ID2	Inhibitor Of DNA Binding 2	3
LCE2B	Late Cornified Envelope 2B	1
LIME1	Lck Interacting Transmembrane Adaptor 1	8
LOC100287896	Uncharacterized LOC100287896	8
LOC101927502	Uncharacterized LOC101927502	8
MMP10	Matrix Metalloproteinase 10 (Stromelysin 2)	1, 4, 5
MRC1	Mannose Receptor C-Type 1	6, 7
MSANTD4	Myb/SANT DNA Binding Domain Containing 4 With Coiled-Coils	8
NFASC	Neurofascin	1
NFIC	Nuclear Factor I C	8
PDPN	Podoplanin	1
PI3	Peptidase Inhibitor 3	1, 4
PLS3	Plastin 3	8
RCHY1	Ring Finger And CHY Zinc Finger Domain Containing 1	2
RNF135	Ring Finger Protein 135	8
RPP38	Ribonuclease P/MRP Subunit P38	8
RUNX3	Runt-Related Transcription Factor 3	8
SLC1A3	Solute Carrier Family 1 Member 3	8
SPP1	Osteopontin	1
TAF6L	TATA-Box Binding Protein Associated Factor 6 Like	8
TFAP2B	Transcription Factor AP-2 Beta	1
ZNF48	Zinc Finger Protein 48	8
ZNF496	Zinc Finger Protein 496	8
ZNF839	Zinc Finger Protein 839	8

1.5. RATIONALE FOR STUDY DESIGN

Treatment with adjuvant radiotherapy modulates immune system in many diseases as witnessed by dynamic changes of humoral and cellular immunity. Moreover, the persistent lymphopenia after radiation therapy is a negative prognostic factor. This study is aimed to explore the changes in immune-cell populations during radiotherapy given as adjuvant treatment for high-risk cutaneous squamous cell carcinomas and to correlate them with patient's outcome.

2. STUDY OBJECTIVES AND OUTCOMES

2.1.1. Primary objective

The primary endpoint is to evaluate the impact of diminished ALC (Absolute Lymphocyte Counts) after RT on patient's outcome.

2.1.2. Primary outcome

Primary outcome consists of disease-free survival (DFS) of patients with ALC < 500 cells/mL after radiotherapy treatment.

2.2.1. Secondary objectives

Secondary objectives include:

- Evaluation of changes (Δ) in the circulating immune-cells population at two time points (before and after RT);
- Correlation between DFS and circulating immune cell population before and after RT;
- Evaluation of changes in immune cell population according to the primary site (h&n vs non-h&n), volume (cc) and administered dose (50 gy; 50-60 Gy; >60 Gy depending on margins involvement) of irradiation. The same parameters will be evaluated according to the nodal basin irradiation in terms of levels included in CTV-HR vs CTV-LR (levels, volume and dose).
- Evaluation of changes (Δ) in the circulating immune-cells population in the subgroup of patients defined as "immunosuppressed" (i.e. patients with chronic hematologic malignant neoplasm, or HIV, or AIDS, or who were treated with immunosuppressive therapies for organ transplantation 6 months or more prior to diagnosis) in respect of non-immunosuppressed patients;
- Evaluation of changes (Δ) in the circulating immune-cells population according to RT induced toxicities;
- 2-year Overall Survival (OS)

2.2.2. Secondary outcome

Secondary outcomes include:

- Changes in white cells blood sub-population defined by the difference between two time points: the day before adjuvant RT (day -1) to day 28 (+/- 3 days) after RT.

In particular, we will evaluate changes of:

- circulating T cells CD3+CD8+ and CD3+CD4+

- absolute lymphocyte count (ALC) and neutrophil counts (ANC) and neutrophil/lymphocyte ratio (NLR)
- Treg lymphocytes, defined as CD4+CD25+FOXP3+ or CD4+CD25hi+CD39+ cells.
- Naïve/Memory T lymphocytes CD4+ and CD8+ (defined as CD45RA/CD45RO)
- myeloid-derived suppressor cells, defined as cells expressing Lin-neg (CD3, CD14, CD15, CD19, CD56) /HLA-DR−/CD33+/CD11b+ in either a "lymphocyte" (small FSCxSSC) gate, or in a "monocyte" (larger FSCxSSC) gate, and as HLA-DR+/lo CD14+ cells in a large gate.
- Plasmacytoid Dendritic Cells (CD303+CD123+)
- NK cells dim (CD3-CD16+CD56+) and bright (CD3-CD16-CD56++);
- NKT cells (CD3+CD56+);
- Myeloid Dendritic cells (CD1c+ and CD141+);
- Monocytes subsets (classical CD14+CD16-, intermediate CD14+CD16+, non classical CD14+/-CD16+);
- B lymphocytes (CD19)
- Analysis of plasma cytokines (TNFalpha, TGFbeta, IL-6, IL-10) by ELISA;
- IFN signature: real-time PCR on mRNA from peripheral blood mononuclear cells (PBMCs)

- DFS of patients according to the values of the above reported immune population at baseline, after RT and according to the changes before/after RT

- Changes in immune population cell at the two time points according to the following variables:
  - Site of irradiation (involving or not nodal basins, head and neck vs non-head and neck area and lymphnodes levels involved in CTV-HR vs CTV-LR)
  - Volume of irradiation (expressed as cc) for primary site and nodal basin (volume received by lymphnodes classified as CTV-HR vs CTV-LR)
  - Dose of irradiation (total dose received in Gy) for primary site (depending on the margin status) and nodal basin (dose received by lymphnodes classified as CTV-HR vs CTV-LR)

- Changes in immune population cells in the immunosuppressed patient subgroup in comparison with non-immunosuppressed patients
- Changes in immune population cells according to RT related toxicities as mucositis and radiodermitis

- 2-year OS

3. SUBJECTS TO BE RECRUITED

3.1. Inclusion criteria

1. Age ≥ 18 years.
2. Signed written informed consent.
3. Histologically confirmed diagnosis of cSCC
4. cSCC categorized as high risk according to ASTRO Guidelines:
    - close or positive margins that cannot be corrected with further surgery (secondary to morbidity or adverse cosmetic outcome)
    - gross perineural spread, as identified by radiological or pathological assessment
    - disease recurrence after a prior margin-negative resection
    - pathological stage T3 and T4
    - desmoplastic or infiltrative tumors in the setting of chronic immunosuppression
    - at pathological exam, cSCC involving regional lymphnodes, with the exception of a single, small (<3 cm) cervical lymph node harboring carcinoma, without extracapsular extension.
5. cSCC addressed to adjuvant radiotherapy as per clinical practice (a complete post-operative treatment should be administered with 50-54 or 60-66 Gy depending on the margin status)
6. Eastern Cooperative Oncology Group (ECOG) Performance status of 0-2.

3.2. Exclusion criteria

1. cSCC not eligible for surgery
2. cSCC not eligible for adjuvant radiotherapy for any condition depending on disease characteristics or patient characteristics, co-morbidities or refusal
3. Any concurrent investigational product, biologic, or hormonal therapy for cancer treatment
4. Concurrent treatment with chemotherapy for the purpose of cSCC cure
5. History or current evidence of any condition that, in the opinion of the treating investigator, might interfere with the subject's participation for the full duration of the trial.
6. Any major surgery, different from that planned for the protocol, in the 15 days before the protocol starting
7. Any radiotherapy treatment in the 28 days before the protocol starting
8. Pregnant or breastfeeding.

NB: patients with chronic hematological malignancies with or without ongoing treatment or patients with autoimmune diseases will be eligible for the study. They will be analyzed both in the whole series of patients and as a separate subgroup.

4. STUDY DESIGN

4.1. Overview

The patients enrolled in the study are affected form cSCC classified at high risk and addressed to surgery and subsequent adjuvant RT according to clinical practice. The aim of the study is to evaluate patients' outcome according to the post-radiation lymphocytes count and to the changes induced in the immune cell population by a loco-regional treatment as radiotherapy.
The way to objectivate these results is to collect some blood samples and analyze them.

The study comprises blood samples collection performed at two time points:

- at baseline (day -1) before adjuvant RT;
- at day 28 (+/- 3) after RT completion.

At any timepoints blood samples will be analyzed for immune cell population detailed in 2.1.2 and 2.2.2 paragraphs.

4.2 Follow up

After the completion of the study analysis at day 28 (+/- 3 days) after radiotherapy, the patients will remain in follow up.
The follow up of will be carried out according to clinical practice, with periodic visits and instrumental assessments, which should performed with the following timepoints:

- Clinical examination every 3 months for the first year, then every 6 months
- Radiological imaging 3 months after RT completion, then every 6 months

Disease free survival of the patients, overall survival, possible side effects and other treatments performed in adjuvant or palliative settings will be collected.

4.3 Duration of study

The duration of the treatment is limited by the completion of adjuvant radiotherapy.
This study will take 24 months to conclude the enrollment of 42 patients.
Follow up of the patients will be carried out according to clinical practice, with periodic visits and

instrumental assessments. Patients' follow-up will continue for 24 months after the end of radiation andpatients still alive will be considered as censored.
Therefore, the whole duration of the study (enrollment + follow up) will be 48 months.

First results will be evaluated in an interim-analyses (primary and secondary objectives of before-after RT change in immune populations) at 24 months.

4.4 Numbers of subjects

Patients with high risk cSCC will be included. The study is a multicentric study and will enroll 42 subjects in Italy.

5. STUDY TREATMENT

5.1. RT technique

Radiotherapy adjuvant treatment will be established during multidisciplinary team discussion taking into account patients and disease characteristics and conducted as per clinical practice. A preliminary visit and a treatment planning will be performed. Multiple RT modalities are allowed in relation to site, dimension and shape of RT field: eg MV (megavoltage) electrons or different techniques using photons (eg Helical or volumetric IMRT; with/without Image guided treatment).
The objective of the RT planning will be obtaining a uniform dose distribution to the target, minimizing the dose to surrounding healthy tissues. Simulation, treatment planning, treatment delivery and verification should be performed according to standard practice.
A high-risk tumor volume for the primary tumor (CTVp_HR) and nodal disease (CTVn_HR) should be recognized in the delineation phase based on all clinical, radiological and pathological findings, enlarged of a 5-mm margin (edited for anatomical barriers) and be targeted to a total dose of 60 Gy at conventional fractionation, whereas those areas deemed at substantial risk of microscopic disease such as positive surgical margin or pathologic lymphnodes with extranodal extension may warrant a boost dose up to 66 Gy (CTVp_HR_Boost or CTVn_HR_Boost).
Adequate information and medication to prevent and managed side effects will be provided during visits and treatment.
The Head and Neck Cancer International Group (HNCIG) Consensus Guidelines (8) should be regarded as the reference paper for the delivery of postoperative RT in high-risk cutaneous squamous cell carcinoma of the head and neck.

5.2. Concomitant medications

With the exception of the prohibited drugs listed in the section 5.3, concomitant drugs are permitted.

5.3. Prohibited Medications

The following medications are prohibited while the subject is in this study:

- Other anti-cancer therapies, except for chronic hematological malignancies
- Investigational/experimental medicines or vaccines
- Other radiotherapeutic treatment in the 28 days before study enrollment

6. ASSESSMENT OF EFFICACY

Blood samples will be collected at two time point to assess immune-modulating effects of adjuvant radiotherapy.

The following blood cells will be assessed at baseline (day -1) before adjuvant RT and at day 28 (+/- 3) after RT completion:

- circulating T cells CD3+CD8+ and CD3+CD4+
- absolute lymphocyte (ALC) count and neutrophil counts (ANC) and neutrophil/lymphocyte ratio (NLR)
- Treg lymphocytes, defined as CD4+CD25+FOXP3+ or CD4+CD25hi+CD39+ cells.
- Naïve/Memory T lymphocytes CD4+ and CD8+ (defined as CD45RA/CD45RO)
- myeloid-derived suppressor cells, defined as cells expressing Lin-neg (CD3, CD14, CD15, CD19, CD56) /HLA-DR−/CD33+/CD11b+ in either a “lymphocyte” (small FSCxSSC) gate, or in a “monocyte” (larger FSCxSSC) gate, and as HLA-DR+/lo CD14+ cells in a large gate.
- Plasmacytoid Dendritic Cells (CD303+CD123+)
- NK cells dim (CD3-CD16+CD56+) and bright (CD3-CD16-CD56++);
- NKT cells (CD3+CD56+);
- Myeloid Dendritic cells (CD1c+ and CD141+);
- Monocyte subsets (classical CD14+CD16-, intermediate CD14+CD16+, non classical CD14+/-CD16+);
- B lymphocytes (CD19+);
- Analysis of plasma cytokines (TNFalpha, TGFbeta, IL-6, IL-10) by ELISA;
- IFN signature: real-time PCR on mRNA from PBMCs

A centralized evaluation of all blood and tissue samples collected will be performed at the Department of Molecular and Translational Medicine, Oncological-Immunological Experimental Section, University of Brescia.

Follow up of all subjects enrolled will be conducted according to clinical practice, with periodic visits and instrumental assessments. Survival status of the patients and possible other treatments performed will be collected for at least 24 months.

Plasma and mRNAs will be stored at the Department of Molecular and Translational Medicine, Oncological-Immunological Experimental Section, University of Brescia. All samples will be identified by a specific numerical code.

7. ASSESSMENT OF ADVERSE EVENTS

Per regulatory requirements, if an event is assessed by the Sponsor Institution as a Serious Unexpected Adverse Reaction (SUSAR), even there is no experimental drug tested in the protocol, it is the responsibility of the Sponsor Institution to submit the SUSAR to Regulatory Authorities according to applicable regulations.

In addition, the SUSAR will be distributed to the Investigators/sites utilizing a Council for International Organizations of Medical Sciences (CIOMS) report form, or the MedWatch 3500A form). The Investigator/site will submit a copy of the report to their respective Institutional Review Board (IRB) or Independent Ethics Committee (IEC) per the governing institutional requirements and in compliance with local laws and guidelines.

8. DATA MANAGEMENT

A full dataset will be collected for all patients enrolled. Qualified study staff will perform collection of the findings derived from the centralized blood samples examination.

8.1 Data confidentiality

Information about study subjects will be kept confidential and managed under the applicable laws and regulations. Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization, the Investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization, attempts should be made to obtain permission to collect follow-up safety information (e.g. has the subject experienced any new or worsened AEs) at the end of their scheduled study period.

8.2 Source Documentation

The Investigator is responsible for ensuring that the source data are accurate, legible, contemporaneous, original and attributable, whether the data are hand-written on paper or entered electronically. If source data are created (first entered), modified, maintained, archived, retrieved, or transmitted electronically via computerized systems (and/or any other kind of electronic devices) as part of regulated clinical trial activities, such systems must be compliant with all applicable laws and regulations governing use of electronic records and/or electronic signatures. Such systems may include, but are not limited to, electronic medical/health records (EMRs/EHRs), adverse event tracking/reporting, protocol required assessments, and/or drug accountability records).

When paper records from such systems are used in place of electronic format to perform regulated activities, such paper records should be certified copies. A certified copy consists of a copy of original information that has been verified, as indicated by a dated signature, as an exact copy having all of the same attributes and information as the original.

8.3 Investigational Site Training

The Sponsor will provide quality investigational staff training prior to study initiation. Training topics will include but are not limited to: GCP, AE reporting, study details and procedure, electronic CRFs, study documentation, informed consent, and enrollment of WOCBP.

Access to the system will be controlled by a sequence of individually assigned user identification codes and passwords, made available only to authorized personnel who have completed prerequisite training.

8.4 Site monitoring

Before study initiation, at a site initiation visit or at an investigator's meeting, Sponsor personnel (or designated CRO) will review the protocol and CRFs with the investigators and their staff. During the study, the field monitor will visit the site regularly to check the completeness of patient records, the accuracy of entries on the CRFs, the adherence to the protocol to Good Clinical Practice, the progress of enrollment, and to ensure that study treatment is being stored, dispensed, and accounted according to specifications. Key study personnel must be available to assist the field monitor during these visits.

The investigator must maintain source documents for each patient in the study, consisting of case and visit notes (hospital or clinic medical records) containing demographic and medical information, laboratory data, electrocardiograms and the results of any other tests or assessments. All information recorded on CRFs must be traceable to source documents in the patient's file.

The investigator must also keep the original signed informed consent form (a signed copy is given to the patient). The investigator must give the monitor access to all relevant source documents to confirm their consistency with the CRF entries. The Sponsor monitoring standards require full verification for the presence of informed consent, adherence to the inclusion/exclusion criteria and documentation of SAEs.

In addition, the study may be evaluated by the Sponsor internal auditors and inspectors from Competent authority who must be allowed access to CRFs, source documents, other study files, and study facilities. the Sponsor audit reports will be kept confidential.

The investigator must notify the Sponsor promptly of any inspections scheduled by regulatory authorities, and promptly forward copies of inspection reports to the Sponsor.

8.5 Data collection

This study will use an Electronic Data Capture (EDC) system. The designated investigator staff will enter the data required by the protocol into the Electronic Case Report Forms (eCRF). The eCRFs have been built using a fully validated secure web- enabled software that conforms to FDA requirements. Investigator site staff will not be given access to the EDC system until they have been trained. Automatic validation programs check for data discrepancies in the eCRFs allow modification or verification of the entered data by the investigator staff.

The Principal Investigator is responsible for assuring that the data entered into eCRF is complete, accurate, and that entry and updates are performed in a timely manner.

8.6 Database management and quality control

The Sponsor personnel (or designated CRO) will review the data entered by investigational staff for completeness and accuracy. Electronic data queries stating the nature of the problem and requesting clarification will be created for discrepancies and missing values and sent to the investigational site via the EDC system. Designated investigator site staff are required to respond promptly to queries and to make any necessary changes to the data. Medical history/current medical conditions and adverse events will be coded using the Medical dictionary for regulatory activities (MedDRA) terminology. The occurrence of any protocol violations will be determined. After the data has been verified to be complete and accurate, the database will be declared locked. Authorization is required prior to making any database changes to locked data, by joint written agreement between the Biostatistics and Data Management and the Sponsor.

8.7 Study governance and oversight

The safety of the study is closely monitored on an ongoing basis by the Sponsor representative and the coordinating investigator in consultation with Sponsor's representatives. Issues identified will be addressed; for instance, this could involve amendments to the study protocol and letters to Investigators.

8.8 Records

8.8.1 Records Retention

The investigator must retain all study records and source documents for the maximum period required by applicable regulations and guidelines, or institution procedures, or for the period specified by the Sponsor, whichever is longer. The investigator must contact the Sponsor prior to destroying any records associated with the study.

The Sponsor will notify the investigator when the study records are no longer needed.

If the investigator withdraws from the study (eg, relocation, retirement), the records shall be transferred to a mutually agreed upon designee (eg, another investigator, EC). Notice of such transfer will be given in writing to the Sponsor.

8.8.2 Case Report Forms

An investigator is required to prepare and maintain adequate and accurate case histories designed to record all observations and other data pertinent to the investigation on each individual treated or entered as a control in the investigation. Data that are derived from source documents and reported on the CRF must be consistent with the source documents or the discrepancies must be explained. Additional clinical information may be collected and analyzed in an effort to enhance understanding of product safety. CRFs may be requested for AEs and/or laboratory abnormalities that are reported or identified during the course of the study.

Electronic CRFs will be prepared for all data collection fields except for fields specific to SAEs and pregnancy, which will be reported on the paper or electronic SAE form and Pregnancy Surveillance form, respectively. Spaces may be left blank only in those circumstances permitted by study-specific CRF completion guidelines provided by the Sponsor.

The confidentiality of records that could identify subjects must be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirement(s).

The investigator will maintain a signature sheet to document signatures and initials of all persons authorized to make entries and/or corrections on CRFs.

The completed CRF, including any paper or electronic SAE/pregnancy CRFs, must be promptly reviewed, signed, and dated by the investigator or qualified physician who is a sub-investigator and who is delegated this task on the Delegation of Authority Form. For electronic CRFs, review and approval/signature is completed electronically through the Sponsor electronic data capture tool. The investigator must retain a copy of the CRFs including records of the changes and corrections.

Each individual electronically signing electronic CRFs must meet Sponsor training requirements and must only access the Sponsor electronic data capture tool using the unique user account provided by Sponsor. User accounts are not to be shared or reassigned to other individuals.

8.8.3 Subject identification – Personal Data protection

All records identifying the subject must be kept confidential and, to the extent permitted by the applicable laws and/or regulations, not be made publicly available. The name of the patient will not be asked for nor recorded at the Data Center. A sequential identification number will be automatically attributed to each patient registered in the study. This number will identify the patient and must be included on all case report forms. In order to avoid identification errors, date of birth will also be reported on the case report forms.

Any and all patient information or documentation pertaining to a clinical trial, to the extent permitting, through a "key" kept anywhere, regardless of whether such key is supplied along with the information or documentation or not, must be considered as containing sensitive personal data of the patient, and is therefore subjected to the provisions of applicable data protection ("privacy") regulations. Breach of such regulations may result in administrative or even criminal sanctions.

Particularly, an information sheet prepared according to such regulations and a form to evidence the consent of patients to the processing of such data must therefore accompany the informed consent administered to the patient. Such information must (i) identify the roles of the holder and processor ("responsible", appointed by the holder) of the patient personal data (also if not directly identifying the patient), as well as the purposes of the personal data collection and processing (medical treatment and related/unrelated scientific research), (ii) adequately describe the flows of communication involving them, particularly if third parties should become involved, and (iii) seek the patient's prior and specific consent to such processing.

Patient information or documentation may be considered "anonymous", and as such not subject to privacy regulations, only when no key whatsoever, permitting the identification of the patient, is any longer available.

Particular attention should therefore be paid (and information/consent materials adapted accordingly) whenever patient data are supplied to third parties and may be autonomously processed, or biological samples/materials are taken and kept for future research purposes, associated or not with the pathology considered in the study.

9. ETHICAL CONSIDERATION

Primary investigator will ensure that this study will be conducted in agreement with either the Declaration of Helsinki (Tokyo, Venice, Hong Kong and Somerset West amendments) or the laws and regulations of the country, whichever provides the greatest protection of the patient.

The protocol will be written, and the study will be conducted according to the ICH Guideline for Good Clinical Practice. The protocol and its annexes will be subject to review and approval by the competent Independent Ethics Committee(s) ("IEC") at each study center.

9.1 Informed consent

The Investigator has both ethical and legal responsibility to ensure that each subject being considered for inclusion in this study is given a full explanation of the study. Written informed consent will be obtained from all subjects (or their guardians or legal representatives) before any study-related procedures (including any pre-treatment procedures, such as pre-procedure sedation) are performed or given. Written informed consent will be documented on an informed consent form (ICF) approved by the same Independent Reviewing Authority (IRA) responsible for approval of this protocol. The ICF will conform to ICH GCP guidelines and to the institutional requirements for

informed consent and applicable regulations. The ICF will be reviewed with the prospective study subject or his or her legal representative, and the Investigator or qualified designee will be available to answer questions regarding procedures, risks, and alternatives. The subject will receive a copy of the signed ICF. The original signed and dated ICF will be kept in the site's regulatory file. Documentation of the subject's informed consent for and participation in this study will be noted in the subject's medical record.

10. STATISTICAL ANALYSIS

https://www.sample-size.net/ (Kohn MA, Senyak J. Sample Size Calculators [website]. UCSF CTSI. 29 April 2021) has been used to calculate the sample size. We consider the expected rate of patients with ALC less than 500 cells/mL being 40% one month after RT end, according to previous works showing this rate being 30% at 3 months in head and neck cancer series treated with RT (18), and the estimated 3-year recurrence-free survival being 36% in patients with ALC less than 500 cells/mL and 63% in patients with ALC greater than 500 cells/mL, so HR=0.45 (JAMA Otolaryngol Head Neck Surg. 2019;145(5):413-421. doi:10.1001/jamaoto.2019.0034).

All statistical analyses will be performed using SPSS version 23.0 (SPSS, Chicago, IL).

10.1 Sample size calculations

To test the Hazard Ratio between Group0 (patients with ALC less than 500 cells/mL) and Group1 (patients with ALC greater than 500 cells/mL) of 0.3 with type I error rate of 5% and power of 80%, we need a minimum of 23 total events which in our hypothesis of median survival time in Group 0 equal to 18 months, 5% censoring rate and whole duration of the study of 4 years correspond to 42 patients, 17 in the Group0 and 25 in the Group1..

10.2 Statistical plan

Disease-free survival and overall survival in treated patients will be analyzed with a Cox proportional hazards regression, first univariable and then multivariable.

Change between T0 (28 days after RT) and T1 (24 months) in secondary endpoints, all recorded as continues variables, will be modelled using linear mixed models (or GEE), accounting for within patient repetition. The interaction between time and ALC status will be the effect of interest. All models will adjust for potentially confounding clinical variables.

.11.SCHEDULE OF EVALUATION

Evaluation	Screening	TREATMENT PERIOD			
		Day -1	RT treatment (period 0)	Day +28 (from the end of RT)	Follow up at 24 months
Informed consent	X				
Inclusion/exclusion criteria	X				
Medical history	X				
ECOG PS Status	X				
Vital signs	X				
Evaluation of high-risk factors for recurrence	X				
RT treatment			X		
RT characteristics (site-volume-dose)			X	X	
Immune markers *		X		X	
Molecular profile		X			
Outcomes: DFS and OS					X

*circulating T cells CD3+CD8+ and CD3+CD4+
absolute lymphocyte (ALC) count and neutrophil counts (ANC) and neutrophil/lymphocyte ratio (NLR)
Treg lymphocytes, defined as CD4+CD25+FOXP3+ or CD4+CD25hi+CD39+ cells.
Naïve/Memory T lymphocytes CD4+ and CD8+ (defined as CD45RA/CD45RO)
myeloid-derived suppressor cells, defined as cells expressing Lin-neg (CD3, CD14, CD15, CD19, CD56) /HLA-DR−/CD33+/CD11b+ in either a “lymphocyte” (small FSCxSSC) gate, or in a “monocyte” (larger FSCxSSC) gate, and as HLA-DR+/lo CD14+ cells in a large gate.
Plasmacytoid Dendritic Cells (CD303+CD123+)
NK cells dim (CD3-CD16+CD56+) and bright (CD3-CD16-CD56++);
NKT cells (CD3+CD56+);
Myeloid Dendritic cells (CD1c+ and CD141+);
Monocytes subsets (classical CD14+CD16-, intermediate CD14+CD16+, non classical CD14+/-CD16+);
B lymphocytes (CD19);

Analyses of plasma cytokines (TNFalpha, TGFbeta, IL-6, IL-10) by ELISA;

IFN signature: real-time PCR on mRNA from PBMCs

Any other treatment performed by the patient in case of progression will be collected and reported in the CRF.

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