ESMO Research Research Fellowship
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FINAL REPORT

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Mentor: Eric Deutsch
Project title: A micro-CT based radiomic approach to identify radio-immune signatures in preclinical models of head and neck and lung cancers
Home Institute: University Hospital of Parma, Via Gramsci 14, 43123 Parma, Italy

Introduction
Radiomics in cancer research and precision medicine
Radiomics is an emerging discipline that converts imaging data into high-dimensional mineable features, with the ultimate goal to generate imaging biomarkers. As a non-invasive process able to assess the tumor and its microenvironment in their entirety, radiomics has the potential to monitor temporal and spatial heterogeneity and immune background. Up to now, limited studies have determined whether tumor immune microenvironment (TIME) could be deciphered through interrogation of radiomic features, ultimately delineating the proneness of cancer patients to respond to treatment. In the era of personalized medicine, where therapeutic strategies are increasingly tailored to patient- and tumor- specific profiles, radiomics might represent a valuable decision support tool, guiding cancer patient management at bedside.

Orthotopic tumours as clinically relevant preclinical models
Murine models of cancer play an essential role in scientific research due to their ability to resemble tumour growth, recapitulating elements and characteristics (i.e. immune contexture) of the human disease and to prove measurable effects of anticancer drugs. Specifically, orthotopic tumour models have been demonstrated to more closely reflect the complexity of human tumour progression and more faithfully recreate the TIME, including tumor cells, surrounding stroma, vasculature, and immune populations.

In vivo micro-CT imaging to track tumor evolution in preclinical models
Micro-CT (µ-CT) approach is emerging as a promising strategy able to longitudinally monitor cancer growth and therapeutic response in preclinical models, whereas histologic examination is a terminal readout. Moreover, murine models, although imperfect from a purely biological point of view, have the advantage of being of small sizes, thus allowing inclusion of all tumors and surrounding tissue for comprehensive histological analysis. This inclusion in its entirety opens a greater possibility of registration of the histological images on the radiological images and of validation of the meaning of the radiomics concept. Data on radiomics in preclinical models are still scarce and just scratched the surface on the establishment of µ-CT-based radiomic pipelines able to decipher tumor immune microenvironmental features.

Rationale and Aim
Research hypothesis
The development of a reproducible µ-CT radiomic platform for orthotopic models of head and neck and lung cancer could represent a fundamental step to decode cancer evolution. Our objective is to carry out a preclinical study on murine models to validate the biological reality of a radiomic-based approach in the evaluation of tumor infiltrating lymphocytes (TILs). The possibility to non-invasively decipher the tumor microenvironment and track treatment response may unveil radio-immune signatures, potentially translatable into clinical practice.

Aims of the Project
The main goal of the present project is to set the basis for a functional imaging of CD8+ TILs density in vivo, which should be useful for basic and preclinical research and drug screening.
Specific aims are the following:
### Aim 1. To develop and optimize a quantitative µ-CT imaging approach in orthotopic mouse models of head and neck and lung cancers.

### Aim 2. To establish and implement a radiomic pipeline able to assess the heterogeneity and biological behaviour of syngeneic orthotopic tumours grafted in immunocompetent mice.

### Aim 3. To comprehensively characterize the immune microenvironment of orthotopic tumour models and evaluate whether µ-CT radiomic features entail specific immunophenotypic profiles.

### Experimental design

#### Study Design

- **Orthotopic Models.** Orthotopic models of head and neck and lung tumours will be established following a methodology previously reported by the INSERM U1030 unit at Gustave Roussy (GR). Animal procedures will be carried out in GR facility, according to protocols approved by the Ethics Committee CEEA26 (EU directive 2010/63/EU).

- **Treatments.** We envision tailored regimens according to emerging biological results. In addition to radiotherapy, monoclonal antibodies directed against specific antigens will be used to selectively enrich or deplete immune populations (i.e. IL-2, anti-TGF-β, anti-CD8 antibodies). This approach, by comparing enriched/depleted vs untreated tumors, will allow to directly identify radiomic signature associated to a given tumor-infiltrating immune cell population. We plan to have up to 50 mice/arm for two experimental arms. We will validate the ability of such radiomic signatures to assess the effects of anticancer treatments as radiotherapy and immunotherapy (as single or combined strategies according to previous protocols).

- **Micro-CT Imaging.** In vivo pre-clinical imaging will be performed using the Quantum FX µ-CT technology (PerkinElmer), available at the Radiobiology of Medical Exposure Laboratory (LRMed), which ensures a useful longitudinal monitoring while maintaining low dose and high-quality images.

- **Immunophenotypic Analysis of TIME.** Tumor samples will be subjected to immunohistochemical (IHC) analysis to evaluate the density and spatial distribution of immune cells (i.e. CD8, CD4, F4/80, etc), followed by image analysis by dedicated software (ImageJ and Delfiniens) at the experimental pathology platform of GR (AMMICa-PETRA). To identify radiomic signatures associated with immune cell infiltration, mice will be euthanized immediately after the acquisition of µ-CT images. A reconstruction algorithm will generate a 3D map of CD8+ TILs. This 3D functional map will be subsequently readjusted to the anatomical volume obtained by the µ-CT.

- **Radiomic analysis.** µ-CT scans will be explored using the pyradiomics python library for feature extraction. After image preprocessing including voxel resampling and image discretization, volumes of interest (VOI) outlining tumor lesions (tumor core and invasive margins) will act as source of information for quantitative analyses at the tumor scale, allowing the extraction of shape, first order and second order features. Different machine learning approaches (lasso, elastic net, SVM, random forest, neural networks) will be tested and compared to train and assess the robustness of radiomic signatures in predicting immune population density (numbers of CD8+ / mm² in the tumor core and at the invasive margin). To go further, we will generate 3D maps of CT texture parameters and perform a voxel-wise correlation analysis (in a broad sense: correlation, logistic regression, advanced machine learning) to validate the biological reality of the extracted parameters. A machine learning analysis will be also performed, given the 3D CT volume as input to the neural network, while the ability to predict the CD8+ TILs 3D map as output.

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**Figure 1.** Schematic representation of our planned study design.
Results, Conclusions and Future Perspectives

Results

▪ Protocol writing and submission to Ethics Committee for Animal Procedures: a detailed protocol reporting all the planned animal experiments was provided to IRSN/SANTE/SERAMED/LRMed-LRAcc Ethics Committee.

▪ Development and optimization of a quantitative μ-CT imaging approach in H&N tumor models (aim 1): we first solved technical issues related to μ-CT detector, thanks to the contribution of expert physics (Prof. Charlotte Robert, Dr. Morgane Dos Santos). Next, we selected the best acquisition and reconstruction protocol able to minimize noise, improve spatial resolution and overcome potential miscalibration errors, such as ring artifacts (Figure 2).

Figure 2. Representative images illustrating our progress in the development of a robust μ-CT imaging approach. On the left, previous μ-CT scans exhibiting a marked ring artifact, on the right the new optimized protocol able to solve this technical issue.

▪ Deployment of a reproducible radiomic pipeline (aim 2): After image pre-processing, including voxel resampling and image discretization, we delineated the volumes of interest (VOI) outlining the entire tumor region (lesion segmentation, see Figure 3), which acted as source of information for quantitative analyses at the tumor scale, thus allowing the extraction of shape, first order and second order features [Pyradiomics library]. Overall, 106 μ-CT based radiomic features have been extracted and will be subsequently correlated with distinct tissue immune contexts.

Figure 3. Example of a tumor segmentation (green) on μ-CT image viewed in 3 different plans: axial (upper left window), coronal (lower left window) and sagittal (lower right window). In the upper right window, 3D rendering of tumor lesion is shown.
Optimization of tissue procedures and IHC staining protocols for H&N tumor models (aim 3): following mice euthanasia and surgical dissection, we collected mice heads (comprising tumors) and fixed in Paraformaldehyde (PFA). Tissue samples were subsequently subjected to a well-established decalcification protocol. A pre-determined region of 7-10 mm, comprising tumor (see Figure 4), was identified and included in paraffin. Tissue sections were cut maintaining 500µm between each slide (Figure 4). The distance was determined based on calculations, contemplating µ-CT image resolution, and will likely enable the subsequent registration of IHC images on µ-CT scans. The IHC staining for CD8+ TILs was conducted on 5µm thick sections by an expert pathologist.

Figure 4. Schematic representation of cutting protocol of tissue samples.

Experiment 1 (H&N tumor models): To determine the exact timeframe of T CD8+ lymphocyte infiltration within the TIME in order to select the optimal timing for radio-immune analyses.

1) Establishment of orthotopic H&N model (N = 30): Injection of 500,000 TC1-luc cells at submucosal site in the right inner lip was performed (Day [D] -7).

2) Randomization and Treatment (D 0): Irradiation (IR): 8 Gy single fraction (2 beams, 10 X 10 mm collimator, SAARP irradiator) + antibody (Ab): intraperitoneal anti TGF-β 800 µg.

Group 1: Treated mice (N = 5) sacrificed at D2 post treatment
Group 2: Treated mice (N = 5) sacrificed at D3 post treatment
Group 3: Treated mice (N = 5) sacrificed at D4 post treatment
Group 4: Untreated mice (N = 5) sacrificed at D2
Group 5: Untreated mice (N = 5) sacrificed at D3
Group 6: Untreated mice (N = 5) sacrificed at D4

3) Flow-cytometric analysis (N = 30): Fluorescence activated cell sorting (FACS) analysis was performed at different time points on cells isolated from enzymatically dissociated tumors.

Results:
As documented by the graphs below (Figure 5), at D4 post treatment the difference in CD8+ T cell infiltration between treated and untreated mice was maximum, with a negligible variation in tumor size. Thus, D4 post treatment was selected as the optimal time for mice euthanasia and subsequent radio-immune analyses.
analyses.

**Figure 5.** Explanatory graphs illustrating T CD8+ density and tumor weight in treated and untreated (control [CTRL]) groups at predetermined time points.

- **Experiment 2 (H&N tumor models):** To confirm the specificity of CD8+ T cell infiltration following IR + aTGF-β treatment and to assess its potential translation into distinct µ-CT extracted radiomic features.

1) **Establishment of orthotopic H&N model (N = 12):** Injection of 500,000 TC1-luc cells at submucosal site in the right inner lip was performed (Day [D] -7).
2) **µ-CT imaging, randomization and treatment (D 0):**
   - µ-CT imaging performed by Quantum FX µ-CT technology (PerkinElmer);
   - IR: 8 Gy single fraction (2 beams, 10 X 10 mm collimator, SAARP irradiator) + Ab: intraperitoneal anti TGF-β 800 μg (N = 10).
3) **CD8+ T cell depletion (D 3):** Intraperitoneal injection of aCD8α antibody 100 μg (N = 5)
   - Group 1 (T cell enriched): IR + aTGF-β (N = 5)
   - Group 2 (T cell depleted): IR + aTGF-β followed by aCD8α (N = 5)
   - Group 3 (control): untreated (N = 2)
4) **µ-CT imaging, euthanasia and samples collection (D 4 - 24 hours after anti-CD8 treatment):**
   - µ-CT imaging performed by Quantum FX µ-CT technology (PerkinElmer);
   - Mice were euthanized by cervical dislocation and the entire heads, comprising tumor, collected and subjected to decalcification protocol → Head inclusion → HES and CD8 IHC staining.

**Results:**
First, we confirmed the efficacy of aCD8 antibody to achieve a substantial T CD8 depletion (**Figure 6, upper panel**). Conversely, in the assumed T cell enriched cases (**Figure 6, lower panel**), the presence of a moderate CD8 infiltration was documented. Intriguingly, in these latter samples, CD8+ T lymphocytes appeared to be preferentially located at the tumor margin, whereas a scarce infiltrate within the tumor core was apparent.
Next, following tumor region segmentation (see Figure 3), we extracted radiomic features (RFs) from µ-CT images collected at D0 and D4, outlining a high variability between and within different treatment groups, although with a slight trend towards RFs clustering (Figure 7).

In view of these two relevant findings, T CD8+ location at tumor margin (a) and high heterogeneity in radiomic features (b), we opted for some adjustments in the study plan.

Therefore, our subsequent experiments foresaw the same treatment strategy, but introducing different tumor
segmentation technique (a) and µ-CT imaging timing (b), in the attempt to intercept distinctive tissue immune features.

- **Experiments 3 and 4 (H&N tumor models – training and validation sets):** To assess the feasibility of a µ-CT based radiomic approach able to identify CD8+ TILs in orthotopic mouse models of head and neck tumor.

1) **Establishment of orthotopic H&N model (N = 40):** Injection of 500,000 TC1-luc cells at submucosal site in the right inner lip was performed (Day [D] -7).
2) **Randomization and Treatment (D 0):** IR: 8 Gy single fraction (2 beams, 10 X 10 mm collimator, SAARP irradiator) + Ab: intraperitoneal anti TGF-β 800 µg (N = 40).
3) **µ-CT imaging, CD8+ T cell depletion (D 3):**
   - µ-CT imaging performed by Quantum FX µ-CT technology (PerkinElmer);
   - intraperitoneal injection of aCD8α antibody 100 µg (N = 20)
   
   **Group 1 (T cell enriched):** IR + aTGF-β (N = 20)
   **Group 2 (T cell depleted):** IR + aTGF-β followed by aCD8α (N = 20)
4) **µ-CT imaging, euthanasia and samples collection (D 4):**
   - µ-CT imaging performed by Quantum FX µ-CT technology (PerkinElmer);
   - Mice were euthanized by cervical dislocation and the entire heads, comprising tumor, collected and subjected to decalcification protocol → Head inclusion → HES and CD8 IHC staining.

Specifically, we opted to perform µ-CT scans at D3, immediately before CD8 depletion, and at D4, before mice euthanasia and tumor sampling. By this approach, we aimed at obtaining more homogeneous and consistent radiomic data from the two different treatment groups, and, more importantly, at comparing pre- and post-treatment RFs (deltaradiomics) from T cell depleted mice to identify treatment-induced textural changes likely reflecting the infiltration of T CD8+ cells.

Moreover, based on the distinct location of T CD8+ lymphocytes within TIME, we adopted a different image segmentation technique which enables to separately consider tumor margin and tumor core (Figure 8), therefore extracting radiomic features from these two distinct regions.

**Figure 8.** Representative figure illustrating our tumor segmentation technique; the tumor core is shown in green, while tumor margins are identified by the yellow ring (0.5 mm thickness).
Conclusion:
So far, we were able to generate a reproducible µCT radiomic platform able to non-invasively decipher TIME features in murine models (aims 1, 2). Exploiting, in finely controlled laboratory settings, the potential of radiomics to timely assess distinct immune parameters and predict treatment outcome, may partly overcome the limitations observed in clinical practice and implement the personalization of therapeutic strategies.

Future perspectives:
In-depth analyses are currently ongoing:
- Extraction of radiomic features from µ-CT scans collected at D3 and D4 from different mice groups (separately considering tumor margin and tumor core);
- Machine-learning analyses of the differential expression of µ-CT derived RFs from T cell enriched and T cell depleted tumors;
- Assessment of RFs at different time points to intercept treatment-induced radiomic changes likely (delta-radiomics);
- Image reconstruction of IHC tissue slides performed by the Artificial Intelligence (AI) team of U1030 to obtain a 3D map of TILs → Registration of reconstructed IHC images over µ-CT scans.

If successful, our multidisciplinary approach may offer the first preclinical “proof of concept” of the biological reality of a radiomic-based approach in the evaluation of tumor infiltrating lymphocytes (TILs).

In the near future, the same experimental procedures might be applied to orthotopic mouse model of lung cancer in order to test and validate the consistency and reproducibility of our identified radio-immune signature.

List of Publications and Presentations Resulting from the Translational Research Project “A micro-CT based radiomic approach to identify radio-immune signatures in preclinical models of head and neck and lung cancers”
Our abstract, entitled “Development of a µCT radiomic platform to identify radio-immune signatures in murine tumor models” and reporting our preliminary data, has been accepted for a Poster Presentation at ESTRO 2023.

So far, in view of the limited timeframe and the necessity to provide robust data, no scientific works on the subject have been published.
We expect to provide more definite data to be presented at next ESMO Congresses (i.e. ESMO 2023) and subsequently published on scientific journals in the field of cancer immunology and immunotherapy (i.e. Journal for ImmunoTherapy of Cancer).

List of Publications and Presentations resulting from other projects during the fellowship period (if applicable)

Publications:

6. Mazzaschi G* and Perrone F*, ... Tiseo M; DETECTION study group. Therapeutic Outcomes and Clinical


Posters

1. G. Mazzaschi, ... M. Tiseo. Exploring blood immune cell dynamics to unravel the immunomodulatory effect of radiotherapy in NSCLC patients undergoing immune checkpoint inhibitors. ESMO Immuno Oncology Congress 2022.


5. G. Mazzaschi, L. Moron Dalla Tor, M. Balbi, G. Milanese, D. Tognazzi, B. Lorusso, F. Trentini, G. Di Rienzo, M. Verzè, M. Pluchino, R. Minari, L. Leo, L. Gnetti, P. Bordi, A. Leonetti, L. Ampollini, G. Roti, F. Quaini, N. Sverzelli, M. Tiseo. Static and dynamic tracking of radiomic and immunophenotypic features predicts the benefit of Immune Checkpoint Inhibitors in advanced NSCLC. ESMO 2022 Congress. This abstract received the Best Poster Award ESMO 2022 (Section: NSCLC metastatic).


8. G. Mazzaschi, ... M. Tiseo. Dynamic profiling of blood immunophenotypes and radiomic features to predict immunotherapy response in advanced Non-Small Cell Lung Cancer. WCLC 2022 Congress.

Selection of Courses and Workshops Attended During the Fellowship

- December 2022: ESMO Immuno-Oncology 2022 [Poster Presenter]
- September 2022: ESMO Congress 2022 [Poster Presenter]
- September 2022: ONCONWEB – Building Bridges to interconnect people (solid tumors, thoracic malignancies, skin cancer, immunotherapy, gastrointestinal tumors, kidney cancer)
- June 2022: ASCO synthesis (COMU event) [Invited Speaker]
Acknowledgements and Personal Statement

At this point of my professional growth, both as medical oncologist and physician, the ESMO Translational Research Fellowship at Gustave Roussy Institute represented a unique opportunity to approach the radio-immune field from a pre-clinical point of view, being involved in new research networks and laying the foundation for amazing future projects.

The scientific results achieved by the Gustave Roussy research team led by Prof. Erich Deutsch have been of considerable significance to advancements in the area of cancer immuno-radiotherapy and radiomic approach. Considering my current engagement in similar works, I felt privileged to have the possibility to join Gustave Roussy Institute to increase my understanding of the basic principles dictating cancer immune surveillance and its clinical implications.

With the support and mentoring of Prof. Eric Deutsch and Dr. Michele Mondini, I carried out several pre-clinical and translational research projects covering different aspects on radio-immuno oncology.

Moreover, the affable and supporting atmosphere in the Unit made this experience unique and extraordinary to me. I highly recommend a fellowship at this institution to every young immuno-oncologist.

SIGNATURES

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<td>Prof. Eric Deutsch</td>
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