Allegato/*Annex B*

PROGETTO DI RICERCA / RESEARCH PROJECT
(max 5 pagine / max 5 pages)

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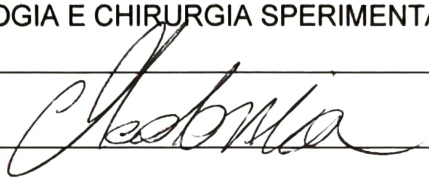
Titolo del progetto / Project title	T-Cell Receptor (TCR) repertoire as predictive biomarker of immune checkpoint inhibitor (ICI) response: a study on baseline features and dynamic evolution across multiple cancer types
Corso di dottorato / PhD	ONCOLOGIA E CHIRURGIA SPERIMENTALI
Firma del candidato/ Applicant's signature	

1 - Abstract

The recent development of immune checkpoint inhibitors (ICIs) has revolutionized oncological treatment [1]. However, despite the identification of various biomarkers predictive of response, many patients still do not respond to immunotherapy [2]. Moreover, the causes of acquired resistance are still not clear [3]. For this reason, the identification of new predictive biomarkers is highly needed. Liquid biopsy allows to easily assess circulating biomarkers directly on peripheral blood instead than on tumor tissue [4]. A novel parameter that can be easily evaluated on peripheral blood and that is recently emerging as a potential predictive biomarker is the T-cell receptor (TCR) repertoire, expression of the number of T-cell clones and of their distribution [5]. Both baseline features of the TCR repertoire and their changes during treatment could represent feasible biomarkers [6]. Additionally, TCR polymorphisms have been associated with the occurrence of treatment toxicity [7]. However, the role of TCR as a predictive biomarker is far from being fully understood, and in addition TCR repertoire appears to behave differently depending on the type of ICI administered [8].
This project aims to evaluate the TCR repertoire of circulation CD8+ T-lymphocytes on peripheral blood through next generation sequencing (NGS) in patients undergoing ICI treatment (independently of tumor histology) divided in subcohorts based on tumor type. We expect to enroll a total of 50 patients. TCR repertoire features will be evaluated at different timepoints to observe possible correlations with disease response and primary and acquired ICI resistance. Primary endpoint will be to establish correlations between TCR baseline features and tumor response. Secondary endpoints will be to study possible correlations between disease response and TCR repertoire changes after one cycle of treatment and at time of disease progression. TCR polymorphisms will also be analyzed to evaluate possible correlations with immune-related toxicities.
This project will help to assess the role of T-lymphocytes in tumor response to ICIs. This could allow to better select patients that may benefit from this treatment and, in future, may help to develop new strategies to overcome tumor resistance.

2 – Project

Background

The role of the immune system in fighting cancer has been known for long [9]. However, it was only in recent years, with the development of ICIs targeting Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), and later programmed cell death receptor 1 (PD-1) and programmed cell death ligand 1 (PD-L1), that immunotherapy really gained ground, revolutionizing cancer treatment [1]. Nowadays, numerous anti-CTLA-4 (e.g. Ipilimumab, tremelimumab) and anti-PD1/PD-L1 (e.g. nivolumab, pembrolizumab, atezolizumab) agents are available, and their use has been approved by international regulatory agencies for multiple cancers, such as non-small-cell lung cancer (NSCLC), small-cell lung cancer (SCLC), melanoma, hepatocellular carcinoma (HCC), renal cancer, gastrointestinal tumors, urothelial carcinoma. Moreover, today these drugs are utilized

not only in monotherapy but also in combination with other ICIs or with other anticancer drugs, such as chemotherapy or tyrosine kinase inhibitors [1].
Immunotherapy often yields durable responses in sensitive patients [3]. However, the introduction of this new class of molecules, with a different mechanism of action compared to standard cytotoxic therapies, has also brought new challenges. Immunotherapy often features atypical or delayed responses, and this has led to the development of specific RECIST (response evaluation criteria in solid tumors) criteria, called immune-RECIST (iRECIST) [10]. Moreover, a significant part of patients that receive ICI therapy do not respond to treatment due to primary resistance [2], and although many molecular biomarkers predictive of response have been proposed and have now entered clinical use (PD-L1 level, tumor mutational burden (TMB), microsatellite instability-high/mismatch repair deficiency (MSI-H/MMRd)) much needs to be done to improve selection of patients that can benefit from this treatment [5] [11]. In addition, at some time, virtually every patient will develop acquired resistance to immunotherapy. However, our knowledge of the mechanisms underlying this phenomenon is limited. Among the supposed causes are neoantigen depletion, defects in antigen presentation and other tumor-intrinsic or extrinsic mechanisms [3].
Some of the main impediments to the clinical use of molecular biomarkers is the lack of tumor tissue: this is often obtained through core biopsy or fine needle aspiration (FNA) and, as such, is usually limited [12]. Moreover, if on one side available specimen is often not recent at the time of ICI treatment start, and thus may not represent the present status of the disease, performing a new biopsy to obtain another sample is not always feasible because of patient's performance status and of the risk of clinical complications [13]. Another limit of tissue biopsy is the inability to depict tumor heterogeneity [14].
Liquid biopsy can be useful to overcome these issues: it is a minimally invasive procedure that evaluate biomarkers through the collection of blood samples and the isolation of circulating tumor components. It can be easily repeated, allowing to account for both temporal and spatial tumor heterogeneity [4]. Various studies have evaluated the role of liquid biopsy in different settings, including the non-invasive evaluation of biomarkers of immunotherapy response, such as PD1/PD-L1 levels and TMB [15].
A promising predictive biomarker for ICI treatment emerging in the last years may be the TCR repertoire [5] [16], which can be defined as the expression of the populations of different T-cell clones within a subject. It is often represented in terms of TCR richness (number of T-cell clones with different TCRs), evenness (relative abundance of different clones) and diversity (which accounts for both richness and diversity) [17]. TCR is a transmembrane receptor of T-cells, responsible of antigen recognition. It is encoded by an alpha and a beta chain, both containing 3 hypervariable regions (CDRs, complementarity-determining regions): while the first 2 are germline-inherited, CDR3 is the result of somatic modifications through V(D)J joining: for this reason, the TCR of every T-cell clone is unique [18]. Sequencing of this region allows to assess the pool of lymphocytes with different TCR, that constitute the TCR repertoire [5] [15]. Although not being a circulating tumor component, this biomarker is easily assessable on circulating lymphocytes from peripheral blood and may provide useful information on the mechanisms of cancer resistance to immunotherapy [19]. Both CD8+ and CD4+ T-lymphocytes have been studied in this setting [15] [20].
However, conflicting data exist on the association between this biomarker and ICI treatment: TCR repertoire appears to behave differently in patients treated with anti-CTLA4 or anti-PD1/PD-L1 agents. Indeed, in a study on melanoma patients, a more clonal TCR repertoire has been linked to longer PFS in patients treated with anti PD1/PD-L1 agents, while on the contrary it has been associated to shorter PFS in patients treated with anti-CTLA4 therapy [8]. Based on these data, another parameter, called TCR convergence, defined as the conversion to a more clonal repertoire during treatment compared to baseline, has been proposed as a possible predictive biomarker for anti-PD1/PD-L1 treatment [6] [21] [22]. Finally, polymorphisms of the TCR beta-chain variable region (TCR variable-beta, TCRVB) have been associated with the occurrence of immune-related adverse events (irAEs) [7].

Primary Objective:
Based on the aforementioned data, the aim of this project will be to study the correlations of baseline features and dynamic evolution of circulating CD8+ T-Lymphocytes TCR repertoire with tumor response to anti-PD1/PD-L1 therapy in patients with metastatic or locally advanced cancer, divided in subcohorts based on tumor histology. TCR repertoire will be analyzed at baseline, at the administration of the second cycle of immunotherapy and at time of tumor progression though a TCR-beta chain long-read NGS assay analyzing all 3 CDRs of TCR beta-chain. This project will provide key insight on the role of T-cells in tumor response to ICIs and may help to better identify patients that could benefit from this treatment.

Secondary objectives:

- To try establishing cutoffs within TCR repertoire features that may discriminate patients between good and bad responders and to study possible differences in TCR repertoire behavior within different cancer types.
- To study potential associations between TCRVB polymorphisms and occurrence of irAEs.

Materials and methods

Primary endpoint:

- Correlation of disease response to ICI treatment (assessed as time to progression (TTP), progression-free survival (PFS), overall survival (OS), overall response rate (ORR)) with circulating CD8+ T-lymphocytes TCR repertoire baseline features (expressed in terms of diversity, richness, evenness, entropy and clonality), studied through peripheral blood analysis, in subcohorts of patients based on tumor histology.

Secondary endpoints:

- Association between the modifications occurring in TCR repertoire after 1 cycle of ICI treatment (such as TCR convergence) and disease response (expressed as TTP, PFS, OS and ORR) in subcohorts of patients based on tumor histology.
- Determination of hallmarks of tumor resistance within TCR repertoire features (diversity, richness, evenness, clonality and entropy) analyzed at time of tumor progression in subcohorts of patients based on tumor histology.
- Identification of TCRVB polymorphisms and correlation with the occurrence of irAEs during ICI treatment.

This project will involve patients with locally advanced or metastatic cancer, about to undergo anti-PD1/PD-L1 treatment. Patients will be continuously enrolled and will be considered eligible independently from tumor type, treatment line and administration schedule. Other inclusion and exclusion criteria are listed below.

Inclusion criteria:

- Signed written informed consent.
- Histologically or cytologically confirmed cancer diagnosis.
- Locally advanced or metastatic cancer.
- Eligibility for treatment with anti PD1/PD-L1 therapy.

Exclusion criteria:

- Prior treatments with immune checkpoint inhibitors.
- Absolute contraindications for ICI treatment.
- Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≥ 3.
- Age <18 years.

Study design

Signed written informed consent will be collected for every enrolled patient. Blood samples will be collected at baseline, at the administration of the second cycle of ICI and at time of tumor progression to isolate CD8+ T-lymphocytes and analyze the features of the TCR repertoire. At every timepoint, for each patient a total of 12 ml of whole blood in four 3-ml EDTA-containing vacutainer tubes will be drawn.

Firstly, density gradient centrifugation will be used to isolate peripheral blood mononuclear cells (PBMCs), using Ficoll-Paque Plus. The obtained pellet will be resuspended in the appropriate buffer and store frozen at -80°C until next use.

Secondly, PBMC will be sorted through flow cytometry to isolate T-lymphocytes and to separate CD8+ from CD4+ T-cells. RNA will be extracted from CD8+ T-lymphocytes through TRIzol-phenol-chloroform RNA purification (Thermo Fisher). RNA will then be quantified using the Qubit 2.0 fluorometer (Thermo Fisher) and the HS Qubit RNA assay kit. RNA integrity assessment will be performed through Agilent 2100 bioanalyzer (Thermo Fisher).

Lastly, the Oncomine TCR beta-LR assay (Thermofisher) will be used to perform NGS analysis of the TCR, following manufacturer's protocol. This assay uses a long-read sequencing technology to sequence all the 3 complementarity-determining regions (CDR1, CDR2 and CDR3) of TCR beta chain, allowing to assess both TCR repertoire and TCRVB polymorphisms. Bioinformatic analysis of the obtained results will be performed through Ion Reporter software to assess parameters such as TCR richness, evenness, clonality (defined as

1- evenness), entropy and Shannon diversity index (both measures of diversity). TCR clonality changes between different timepoints, such as the occurrence of TCR convergence, will also be assessed.
Disease response will be assessed in terms of TTP, PFS, OS and ORR. To do this, data from radiological evaluations will be recorded to assess disease status at baseline and throughout treatment, until disease progression.
IrAEs will be assessed through all the duration of ICI treatment according to Common Terminology Criteria for Adverse Events version 5.0 (CTCAE v 5.0).
Baseline clinical and biological features will be evaluated for all enrolled patients: ECOG PS, presence of comorbidities, levels of circulating markers (e.g. LDH, CEA, CA19.9), neutrophils and lymphocyte count and neutrophile to lymphocyte ratio (NLR). These parameters will be also reassessed during treatment.

Statistical analysis
A total of 50 patients are expected to be enrolled in this study. This number is appropriate to the available timeline and logistics.
Data concerning clinical features of enrolled patients will be analyzed and described as mean and standard deviation or median and interquartile range.
TTP will be calculated as time from beginning of treatment to disease progression, last follow up (censored) or death by any cause (censored); PFS as time from beginning of anti-PD1/PD-L1 treatment to disease progression or death by any cause or last follow up (censored); OS from beginning of treatment to death by any cause or last follow up (censored); ORR as the proportion of patients that will obtain a partial or complete response according to iRECIST criteria. The analysis of PFS, TTP and OS will be performed using the Kaplan-Meier method. IrAEs will be analyzed and described according to CTCAE v5.0.
TCR repertoire features at each timepoint will be assessed through the analysis of richness, evenness, entropy, Shannon Diversity Index and clonality, using the Ion Reporter software. Changing in TCR repertoire and TCR convergence within the same patient through time will be evaluated by studying the proportion of overlapping clones between timepoints using Jaccard index. Spearman correlation coefficient will be used to compare the features of each repertoire.
Univariate and multivariate Cox proportional hazard regression models will be built in order to assess the predictive role of TCR repertoire and to identify independent predictive factors for PFS.
In addition, receiver operating characteristics (ROC) analysis will be performed and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of baseline TCR features will be analyzed, aiming to explore possible thresholds that may stratify patients between good and bad responders.
The comparison between subgroups will be performed using Pearson's chi-square test and Anova test.
All tests will be performed with a significance level of $p = 0.05$. Statistical analyses will be conducted using IBM SPSS Statistics for Windows Version 27.0 (IBM Corporation, Armonk, NY, USA).

Timeline

Work packages (WP)	Activities
Work package 1	Scientific literature update
Work package 2	Patients' enrollment
Work package 3	Sample collection
Work package 4	Bioinformatic analysis
Work package 5	Statistical analysis
Work package 6	Article Publication

Years	Year 1												Year 2												Year 3											
Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Scientific literature update																																				
Patients' enrollment																																				
Sample collection																																				
Bioinformatic analysis																																				
Statistical analysis																																				
Article Publication																																				

Expected results

Immunotherapy has revolutionized the landscape of cancer treatment, dramatically improving outcomes for responding patients, and often producing durable responses. However, a significant subset of patients does not respond to immunotherapy. Available biomarkers (PD-L1, TMB, MSI) are still unable to comprehensively predict response and tissue biomarker analysis is often unfeasible. For these reasons, new predictive biomarkers that can be reliable and easily evaluated on peripheral blood, are highly needed.
In this setting, this project will help to better define the role of CD8+ T-lymphocytes in the response to anti-PD1/PD-L1 treatment, and to link specific TCR repertoire features to the occurrence of primary or acquired resistance. Moreover, the study of TCRVB polymorphisms could help identifying patients at higher risk for irAEs.
This project could allow to better select those patients that may benefit the most from ICI treatments, improving disease management and clinical decision making. Moreover, the uncovering of TCR repertoire features associated to resistance to ICI treatments may allow, in future, to study and develop new therapeutic strategies that could overcome tumor resistance mechanisms.

3 - References

[1] C. Robert, "A decade of immune-checkpoint inhibitors in cancer therapy," (in eng), *Nat Commun,* vol. 11, no. 1, p. 3801, 07 2020, doi: 10.1038/s41467-020-17670-y.

[2] T. Seto, D. Sam, and M. Pan, "Mechanisms of Primary and Secondary Resistance to Immune Checkpoint Inhibitors in Cancer," (in eng), *Med Sci (Basel),* vol. 7, no. 2, Jan 2019, doi: 10.3390/medsci7020014.

[3] B. Zhou, Y. Gao, P. Zhang, and Q. Chu, "Acquired Resistance to Immune Checkpoint Blockades: The Underlying Mechanisms and Potential Strategies," (in eng), *Front Immunol,* vol. 12, p. 693609, 2021, doi: 10.3389/fimmu.2021.693609.

[4] E. Augustus *et al.*, "Prognostic and Predictive Biomarkers in Non-Small Cell Lung Cancer Patients on Immunotherapy-The Role of Liquid Biopsy in Unraveling the Puzzle," (in eng), *Cancers (Basel),* vol. 13, no. 7, Apr 2021, doi: 10.3390/cancers13071675.

[5] G. T. Gibney, L. M. Weiner, and M. B. Atkins, "Predictive biomarkers for checkpoint inhibitor-based immunotherapy," (in eng), *Lancet Oncol,* vol. 17, no. 12, pp. e542-e551, Dec 2016, doi: 10.1016/S1470-2045(16)30406-5.

[6] J. Cham *et al.*, "Combination immunotherapy induces distinct T-cell repertoire responses when administered to patients with different malignancies," (in eng), *J Immunother Cancer,* vol. 8, no. 1, 05 2020, doi: 10.1136/jitc-2019-000368.

[7] T. Looney, E. Linch, G. Lowman, L. Miller, J. Zheng, and D. Topacio-Hall, "Evaluating the link between T cell receptor beta variable gene polymorphism and immune mediated adverse events during checkpoint blockade immunotherapy.," vol. 36, no. 15_suppl, ed. Journal of Clinical Oncology, 2018.

[8] S. A. Hogan *et al.*, "Peripheral Blood TCR Repertoire Profiling May Facilitate Patient Stratification for Immunotherapy against Melanoma," (in eng), *Cancer Immunol Res,* vol. 7, no. 1, pp. 77-85, 01 2019, doi: 10.1158/2326-6066.CIR-18-0136.

[9] M. Abbott and Y. Ustoyev, "Cancer and the Immune System: The History and Background of Immunotherapy," (in eng), *Semin Oncol Nurs,* vol. 35, no. 5, p. 150923, 10 2019, doi: 10.1016/j.soncn.2019.08.002.

[10] D. Ippolito *et al.*, "Immune response evaluation criteria in solid tumors for assessment of atypical responses after immunotherapy," (in eng), *World J Clin Oncol,* vol. 12, no. 5, pp. 323-334, May 2021, doi: 10.5306/wjco.v12.i5.323.

[11] C. Pilard, M. Ancion, P. Delvenne, G. Jerusalem, P. Hubert, and M. Herfs, "Cancer immunotherapy: it's time to better predict patients' response," (in eng), *Br J Cancer,* Jun 2021, doi: 10.1038/s41416-021-01413-x.

[12] L. Pasini and P. Ulivi, "Liquid Biopsy for the Detection of Resistance Mechanisms in NSCLC: Comparison of Different Blood Biomarkers," (in eng), *J Clin Med,* vol. 8, no. 7, Jul 2019, doi: 10.3390/jcm8070998.

[13] F. Pesapane *et al.*, "Will traditional biopsy be substituted by radiomics and liquid biopsy for breast cancer diagnosis and characterisation?," (in eng), *Med Oncol,* vol. 37, no. 4, p. 29, Mar 2020, doi: 10.1007/s12032-020-01353-1.

[14] M. Ilié and P. Hofman, "Pros: Can tissue biopsy be replaced by liquid biopsy?," (in eng), *Transl Lung Cancer Res,* vol. 5, no. 4, pp. 420-3, Aug 2016, doi: 10.21037/tlcr.2016.08.06.

[15] P. Hofman, S. Heeke, C. Alix-Panabières, and K. Pantel, "Liquid biopsy in the era of immuno-oncology: is it ready for prime-time use for cancer patients?," (in eng), *Ann Oncol,* vol. 30, no. 9, pp. 1448-1459, 09 2019, doi: 10.1093/annonc/mdz196.

[16] S. Valpione *et al.*, "Immune-awakening revealed by peripheral T cell dynamics after one cycle of immunotherapy," (in eng), *Nat Cancer,* vol. 1, no. 2, pp. 210-221, Feb 2020, doi: 10.1038/s43018-019-0022-x.

[17] I. Kirsch, M. Vignali, and H. Robins, "T-cell receptor profiling in cancer," (in eng), *Mol Oncol,* vol. 9, no. 10, pp. 2063-70, Dec 2015, doi: 10.1016/j.molonc.2015.09.003.

[18] S. M. Lewis, "The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses," (in eng), *Adv Immunol,* vol. 56, pp. 27-150, 1994, doi: 10.1016/s0065-2776(08)60450-2.

[19] S. Sau and A. K. Iyer, "Immunotherapy and molecular role of T-cell in PD-1 antibody treated resectable lung cancer patients," (in eng), *J Thorac Dis,* vol. 10, no. 8, pp. 4682-4685, Aug 2018, doi: 10.21037/jtd.2018.07.66.

[20] M. Ahmadzadeh *et al.*, "Tumor-infiltrating human CD4," (in eng), *Sci Immunol,* vol. 4, no. 31, 01 2019, doi: 10.1126/sciimmunol.aao4310.

[21] E. Naidus *et al.*, "Early changes in the circulating T cells are associated with clinical outcomes after PD-L1 blockade by durvalumab in advanced NSCLC patients," (in eng), *Cancer Immunol Immunother,* vol. 70, no. 7, pp. 2095-2102, Jul 2021, doi: 10.1007/s00262-020-02833-z.

[22] A. C. Hopkins *et al.*, "T cell receptor repertoire features associated with survival in immunotherapy-treated pancreatic ductal adenocarcinoma," (in eng), *JCI Insight,* vol. 3, no. 13, 07 2018, doi: 10.1172/jci.insight.122092.