ALLEGATO B

Bando 2020-21 - Programma 5 per mille anno 2018-2019
Investigator Grant (IG)

TRANSLATIONAL RESEARCH

LILT will support research projects in the field of cancer aimed at improving cancer diagnosis and treatment. Particularly considered will be those translational research projects that promise short-medium term effects in clinical practice, concerning new diagnostic methodologies and new therapies. Multicentric studies with national coordination, aimed at validating new diagnostic methods, diagnostic, prognostic and predictive tumor markers, able to improve the clinical management of cancer patients are potentially eligible for funding. Specific research projects on new oncological therapeutic approaches are also eligible for LILT funding as IG. For this type of grants it is necessary to demonstrate solid preliminary experimental data supported by a rigorous biological rationale.

1. Principal investigator's full name and qualification:

Prof. **MATTEO LANDRISCINA**, Full Professor of Medical Oncology, Department of Medical and Surgical Sciences, University of Foggia. Principal Investigator of the Solid tumor research group, IRCCS-CROB of Rionero in Vulture.

P.I.'s CV is attached as Supplementary Document 1. The Collaborative Agreement between the University of Foggia and the IRCCS-CROB is provided as Supplementary Document 1b.

2. Proposal title: Targeting metabolic dysregulation to bypass immune escape in human colorectal carcinoma.

3. Primary area of Relevance: Translational oncology research on colorectal cancer

4. Relevance for the National Health System: The working hypothesis of this research proposal to target metabolic alterations/pathways in colorectal cancer cells to improve the activity of immune therapy and revert immune escape mechanisms represents a major objective in the clinical managements of metastatic colorectal cancer patients. The achievement of this goal is a step towards personalized medicine in the treatment of this disease.

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14.01.2021
18:50:36 UTC

7. Proponent's signature Prof. Matteo Landriscina

8. Authorized Administrative Official's signature Prof. Pierpaolo Limone

9. Place and date: Foggia, 14/01/2021

SELF EVALUATION FORM

1. Investigator's full name: (PI) Prof. Matteo Landriscina
2. Total papers: 130; IF: 514.3
3. Total papers (last 10 years): 69; IF: 302.7
4. Total Papers as first/last author or corresponding author: 83
5. Total H-index: 33

PROPOSAL MAIN BODY

1. **Proposal title: TARGETING METABOLIC DYSREGULATION TO BYPASS IMMUNE ESCAPE IN HUMAN COLORECTAL CARCINOMA**
2. **Abstract**

Pembrolizumab was recently established as a new standard of care for patients with MSI-H metastatic colorectal cancer (mCRC), being, conversely, inactive in the vast majority of human CRCs with MSS status. Thus, novel strategies to improve/expand the activity of immune checkpoint inhibitors (ICI) are needed. Among several mechanisms of immune tolerance, metabolic rewiring is gaining consideration as a strategy used by cancer cells to escape the immune response. Indeed, metabolic remodeling induces changes of gene expression through epigenetic and post-transcriptional regulation and modifies the tumor microenvironment, thus creating a loop in which the modified microenvironment induces further metabolic alterations and/or sustains aberrant metabolism. In such a perspective, this interplay between cancer metabolic remodeling and gene expression reprogramming sustains immune escape mechanisms. According to the recent Colorectal Cancer Subtyping Consortium, CRC is classified into four consensus molecular subtypes (CMSs) with distinguishing biologic features, being the CMS1 hypermutated, frequently MSI and with higher likelihood to respond to ICI and the CMS3 epithelial, with evident metabolic dysregulation and unresponsiveness to ICI.

The present study aims at demonstrating that the differences between immune-responsive (CMS1) and immune-resistant (CMS3) cancer cells are, at least in part, due to metabolic alterations taking place in immune-resistant cells. To this aim we will:

- identify metabolic differences between CMS1 and CMS3 cell tumors and the relevant metabolites characterizing tumor microenvironment;
- characterize the molecular mechanisms underlying the above-mentioned metabolic alterations, by exploring the epigenetic and the gene expression profile of the two tumors subtypes at DNA and mRNA levels, focusing on the enzymes and signaling mechanisms likely involved in relevant metabolic pathways;
- explore the hypothesis that metabolic alterations may affect gene expression programs at transcriptional and/or post-transcriptional levels, thus inducing a vicious loop, which progressively worsen the immune resistance;
- explore the effect of tumor metabolic modulation on the immune response *in vitro* and in immunodeficient mice engrafted with human CMS3 CRCs.

These analyses will allow to understand whether the correction of metabolic alterations is

able to reduce the immune resistance and to identify new epigenetic/metabolic pathways as potential targets for therapeutic approaches aimed at reverting immune escape mechanisms. This will be achieved by novel multiomics and traditional cell and molecular biology approaches and using *in vitro, ex vivo* and *in vivo* human CRC cell models.
The novel experimental approach described in this proposal and the validation of metabolic regulation in intra- and inter-cellular signaling adaptation will help to achieve milestones towards personalized medicine.

3. Introduction

Colorectal cancer (CRC) is a frequently lethal disease with heterogeneous outcomes and drug responses. A major reason of the unsatisfactory outcome of metastatic CRC (mCRC) is the inadequate response to pharmacological therapy. This is due to complex mechanisms of drug resistance that interact with each other, rendering CRC cells strongly refractory to the available pharmacological regimens based on conventional chemotherapy, molecular-targeted agents, and, more recently, immune checkpoint inhibitors (ICI). Regarding ICI, clinical studies suggest that their activity is restricted to Microsatellite Instability High (MSI-H) mCRCs. Recently, the phase III KEYNOTE-177 study evaluated efficacy and safety of pembrolizumab *versus* standard chemotherapy as first-line therapy for MSI-H mCRC. Pembrolizumab was superior to chemotherapy for progression free survival (PFS) with fewer treatment-related adverse events (AEs), suggesting that pembrolizumab is a new standard of care for MSI-H mCRC patients (1).
It is important to note that the vast majority of mCRC patients are Microsatellite Stable (MSS) and, thus, unresponsive to checkpoint inhibitor therapy. Thus, novel strategy to improve/extend the clinical activity of these agents are needed. In such a perspective, a huge effort has been done to find a consensus about CRC classification that would help to assign more tailored therapies. The Colorectal Cancer Subtyping Consortium classified CRCs into four consensus molecular subtypes (CMSs) with distinguishing biologic features: CMS1 (microsatellite instability immune, 14%), hypermutated, MSI and with strong immune activation; CMS2 (canonical, 37%), epithelial, marked with WNT and MYC signaling activation; CMS3 (metabolic, 13%), epithelial and with evident metabolic dysregulation; and CMS4 (mesenchymal, 23%), with activation of prominent transforming growth factor–β (TGF-β), stromal invasion and angiogenesis, matrix remodeling pathways and the complement-mediated inflammatory system *(2*). CMS1 encompasses the majority of MSI-H tumors. They are hypermutated, have overexpression of proteins involved in DNA damage repair consistent with defective DNA mismatch repair, low prevalence of somatic copy number alterations, a widespread hypermethylation status, and frequent occurrence of BRAF mutations. Furthermore, CMS1 tumors are characterized by increased expression of genes associated with a diffuse immune infiltrate, along with strong activation of immune evasion pathways, an emerging feature of MSI CRC. CMS2–CMS4 display higher chromosomal instability, but, among those, CMS3 samples had a distinctive global genomic and epigenomic profile and show overrepresentation of KRAS mutations, that have been described as inducing prominent metabolic phenotype.

4. Background and Rationale

Metabolic alterations of tumor cells have the ability to remodel the tumor microenvironment and create an immune-permissive environment. Altered cancer cell metabolism induces changes of gene expression through epigenetic modifications and post-transcriptional regulation and modifies the tumor microenvironment, thus creating a loop in which the modified microenvironment induces further metabolic alterations and/or sustains aberrant metabolism (3). Indeed, cancer metabolism and transcriptional regulation are intimately linked, since they can influence each other by changing the availability of metabolites that act as “donors” for epigenetic modifications, e.g. acetyl or methyl groups for histone acetylation and DNA/histone methylation, respectively, and hence impacting on the expression of several genes, including metabolic and/or

epigenetic-related players (4). In addition, metabolic alteration can influence the activity of enzymes involved in DNA and histone marks regulation, i.e. “writers”, “readers” or “erasers”, thereby regulating acetylation, DNA methylation at promoter-associated CpG islands, correlated with gene silencing and/or on specific histone residues (5). It has been extensively reported that aggressive cancer cells may display severe alteration in the tricarboxylic acid (TCA) cycle resulting in the abnormal accumulation of intermediates such as succinate, fumarate and D-2-hydroxyglutarate. These molecules, also termed oncometabolites, compete with α-ketoglutarate-dependent dioxygenases, including the ten eleven translocation (TET) family of DNA demethylating enzymes and the JmjC domain-containing histone lysine demethylases (JMJDs), with a clear impact on the chromatin methylation profile (6).

Cancer cells escape immune surveillance and develop resistance to immunotherapy by acquiring genetic alterations; consequently, some patients exhibit primary or acquired resistance to novel immune therapies. Recent advancements in the understanding of the mechanism of cancer immune escape suggest that metabolic reprogramming participates to further genetic, epigenetic and signaling pathway modifications, thus remodeling gene expression to sustain immune escape. In such a context, several metabolic pathways have been identified as potentially responsible for inducing immune tolerance in cells infiltrating tumor microenvironment (3). As paradigmatic examples, lactate produced by the increased aerobic glycolysis obstructs the immune response, as well as the accumulation of tryptophan metabolites such as kynurenine paralyzes cancer-killing immune cells and expands the populations of suppressive immune cells in the tumor microenvironment, contributing to immune escape and malignancy. Accordingly, many cancers express high levels of the enzymes involved in the conversion of tryptophan to kynurenine, TDO2 and IDO1, and high tumor IDO1 expression is associated with poor patient outcomes (3). Thus, the crosstalk between metabolism and gene expression reprograming and its effect on tumor microenvironment and acquisition of immune escape mechanisms represent a relevant and novel field of research. In such a context, novel approaches to assess the role of metabolic dysregulation in immune tolerance are expected to improve our ability to target metabolic reprogramming and in turn improve the efficacy of immunotherapy in mCRC.

The present study aims at demonstrating that the differences between immune-responsive (CMS1) and immune-resistant (CMS3) cells are, at least in part, due to metabolic alterations taking place in immune-resistant cells. To this aim we will:

- identify metabolic differences between CMS1 and CMS3 cell tumors and the relevant metabolites characterizing tumor microenvironment;
- characterize the molecular mechanisms underlying the above-mentioned metabolic alterations, by exploring the epigenetic and the gene expression profiles of the two tumors subtypes at DNA and mRNA levels, focusing on the enzymes and signaling mechanisms likely involved in relevant metabolic pathways;
- explore the hypothesis that metabolic alterations may affect gene expression programs at transcriptional and/or post-transcriptional levels, thus inducing a vicious loop, which progressively worsen the immune resistance.

The achievement of these goals will allow us to identify possible new targets for therapeutic approaches aimed at reverting immune escape mechanisms.

5. **Experimental design**

In immune-sensitive (CMS1) *versus* immune-resistant, metabolic (CMS3) CRC cells we will perform:

- Metabolic analyses to assess the metabolic profiles of the two systems and to identify relevant metabolites enriched/depleted in one of the two models (**Task 1**);
- Expression profiling at transcriptional level to identify the genes accompanying

metabolic alterations, focusing on the enzymes and signaling mechanisms involved in the pathways identified in Task 1. Ribosome profiling-Sequencing to assess at translation level the modification of gene expression and to identify changes in mRNA translation associated with metabolic alterations. Analysis of DNA methylation accompanying metabolic remodeling in tumor cells (**Task 2**);

- Treatment of the two cell models with compounds known to affect metabolic/epigenetic pathways emerging for the results of Tasks 1 and 2, to understand whether the correction of the metabolic defects in CMS3 could modify, at least in part, the expression profile and/or the epigenetic alterations accompanying the metabolic alterations (**Task 3**).
- Exploring the effects of specific metabolites, or the treatment of CMS1 or CMS3 cells with the compounds identified in Task 3, on cells of the immune system which control cancer cell survival and proliferation, to address if the correction/induction of metabolic alterations have any effects of the immune response (**Task 4**);
- The "metabolic signature" identified will be validated *in vivo*. Compounds targeting the identified metabolic/gene expression pathways will be tested in humanized mouse models (**Task 5**).

Task1. Metabolomic analysis. In order to assess the metabolic features of the two CRC subtypes and identify the relevant metabolites enriched or depleted in one of the two models, a selected panel of CMS1 immune-responsive (HCT116, RKO, SW48) and CMS3 "metabolic", immune-resistant (LS-180, CL-40, LS-513) cells lines will be subjected to metabolic characterization using (i) Seahorse analysis; (ii) high-resolution respirometry using the Oroboros O2k system for the combined measurement of OXPHOS, mitochondrial membrane potential, ATP production and pH; (iii) analysis of metabolic pathways by biochemical assays (i.e. enzymatic activity, expression analysis of metabolic enzymes, by western blot). In order to strengthen these data, in parallel we will perform an *in situ* hybridization - RNAscope and immunohistochemistry (IHC) - on tissue explants derived from CMS1 and CMS3 CRC-bearing patients. These analyses will be improved by a steady-state GC- and LC-mass spectrometry (MS) analysis of intracellular (metabolome) and extracellular metabolites (exometabolome) of both cell models. By this untargeted metabolomic analyses, metabolites with differing abundance in immune-responsive CMS1 subtype with respect to the immune-resistant CMS3 subtype will be identified, which provides an initial correlation of the metabolome with the phenotype. By a bioinformatics-based analysis, the identified metabolites will be assigned to known metabolic network through the interrogation of metabolic pathway databases (MetExplore). Protein and gene expression data, obtained by multiomic analyses (see Task 2), will be used to support the metabolic networks identified by the *in-silico* approach.

Task 2. Multiomic analysis. We expect to identify the regulatory complex network elicited by cancer metabolic remodeling and the bidirectional interplay between metabolic and gene expression reprogramming. This will provide the prerequisite to understand biological processes driving tumor cell response to immune therapy. Thus, metabolic data obtained in Task 1 will be integrated in a multiomic approach by combining datasets that measure different levels of gene expression. Immune-responsive (CMS1) and immune-resistant (CMS3) cells will be comparatively studied for epigenetic modifications, mRNA expression and translation, to support a role for a transcriptional and post-transcriptional integrated control of cancer cell phenotype upon metabolic remodeling and to identify novel gene networks/pathways involved in metabolic control regulated by the transcriptional mechanisms.

This will be realized through integration of three approaches:

a) DNA methylation profiling. We will perform global gene methylation analysis of CMS1 and CMS3 cell lines, which will allow us to identify genes potentially

methylated/repressed through differential accumulation of metabolites identified in Task 1.

b) Gene expression profiling. Whole genome gene expression analysis through RNA-sequencing will allow the identification of globally enriched pathways in CMS3 cells compared to CMS1, and to match mRNA expression levels to the corresponding methylation status.

c) Ribosome profiling (RP). The expression levels obtained by RP define what is called translatome. Translatome estimations of gene expression levels correlate with proteomic data much better than transcriptome-derived estimations (7). Therefore, this approach will show proof of the final outcome of the gene expression changes accompanied by metabolic alterations. Even more importantly, RP also provides estimations of the mRNAs translation rates, of its regulation and non-canonical functions, providing independent information about alteration of expression that occur at post-transcriptional level.

The statistical integration of genomic, transcriptomic and metabolomic data will be performed using pathway enrichment, biological network or empirical correlation analysis by IntegrOmics platform.

As next step, data from the above multiomic analyses will be validated in human CMS1 and CMS3 CRCs. A cohort of about 200 human CRCs at different stages and already profiled for CMS status is available from the Tumor Tissue Biobank of the IRCCS-CROB. CMS1 and CMS3 tumors will be used to validate metabolomics data from Task1 and gene expression and pathway/network data from Task 2. Immunohistochemistry, western blot and quantitative RT-PCR (RT-qPCR) analyses will be performed on tumor specimens and ELISA to evaluate specific metabolites on plasma from the same patients. As further strategy to validate epigenetic data obtained from previous studies, we expect to use our cohort of human primary-resistant and sensitive CRCs already profiled for global gene methylation. In such a context, gene methylation allowed to select a subgroup of human mCRC with poor prognosis, CpG island methylator phenotype (CIMP)-high and MSI-like phenotype. This MSI-like/CIMP high cohort will be further analyzed (in comparison with the MSS/CIMP-low cohort) for CMS subtypes and used for the validation of metabolic/epigenetic data. Public datasets of human CRCs will be also used for this analysis. Pathways and metabolites validated through this approach will be object of further investigation in subsequent Tasks.

Task 3. Crosstalk between metabolism and gene expression. This task will be dedicated to dissect the molecular mechanism responsible for the relationship between metabolic rewiring and gene expression reprogramming in CMS1 *versus* CMS3 cell lines and to understand how this may modify the immune phenotype of cancer cells. We expect to evaluate the hypothesis that the metabolic phenotype of CMS3 cell lines is responsible for the production/accumulation of specific intermediate metabolites, which favor the remodeling of DNA methylation and gene expression and this is, in turn, responsible for the immune phenotype of the corresponding tumor. Indeed, it is known that specific intermediate metabolites modulate the activity of enzymes involved in DNA remodeling and most of them are strictly connected with glycolysis and TCA cycles (6). Furthermore, metabolic remodeling affects gene expression at multiple levels: by inhibiting demethylation/expression of clusters of miRNAs that, in turn, reduce post-transcriptionally the expression of their targets (8), by modulating the activity of the mTOR, a pathway that is central to protein synthesis (9) and by diverting the activity of metabolic enzymes (10). In such a context, we expect to validate the dependency of DNA methylation and gene expression remodeling on single metabolic pathways by using selective metabolic inhibitors and/or genetic manipulations or by supplementation of cell cultures with intermediate metabolites. Conversely, the inhibition of DNA methylation will be evaluated as strategy to reprogram gene expression and tumor cell metabolism and, consequently, the immune phenotype. Metabolites, metabolic and epigenetic inhibitors will be selected based on data from Tasks 1 and 2, and used for the validation of multiomic data. RNA-

seq data will be validated by RT-qPCR, methylation profiling will be explored through ChIP assay, while RP data will be validated through polysome profiling followed by extraction of RNAs from the actively translating polysomal fraction and RT-qPCR. The association of relevant metabolic enzymes of the highlighted pathways to the translational machinery in the presence/absence of the identified metabolites/compounds will be verified by extraction of polysome-associate proteins and subsequent Western blot.

Task 4. Crosstalk between metabolism and immune phenotype. After the metabolic and gene expression profiling of CMS1 and CMS3 CRC cell lines and the identification of compounds able to either correct the metabolic alterations observed in CMS3 cells, we aim at exploring the effect of tumor metabolic modulation on the immune response. These analyses will allow to understand whether the correction of metabolic alterations is able to reduce the immune resistance and to improve the efficacy of ICI. This aim will be pursued through the evaluation of effects of cancer cell metabolic and gene expression reprogramming on immune cells. CMS1 and CMS3 cell lines, pre-treated or not with compounds identified by Task 3 will be cultured with peripheral blood mononuclear cells (PBMCs) purified from healthy donors in transwell plates. Cell supernatants will be collected for the Luminex assay to evaluate cytokines production and soluble forms of proteins that play a crucial role in the control of the antitumor immune response. In parallel experiments, the transwell inserts will be removed and PBMCs will be used for a FACS polychromatic flow cytometry immunophenotyping. In addition, to test whether the metabolic modifications induced in CMS1 and CMS3 cell lines are able to modify the migratory capacity of immune cells, we will perform a transwell migration assays. PBMCs from healthy donors will be stained with CellTracker™ green Dye, added to a transwell plate and allowed to migrate overnight towards CMS1 and CMS3 cell lines, pre-treated or not with compounds able to modify cell metabolism identified in Task 3.

Task 5. *In vivo* validation in humanized mouse models. To explore the *in vivo* relevance of our *in vitro* findings, we will use the humanized mice model engrafted with fresh human colorectal tumor tissues and reconstituted with PBMC from the same patients from whom the xenografts were derived. Humanized mice with functional human immune systems represent a powerful model for understanding the interaction between human immune components and human cancer and could be a useful tool to evaluate whether metabolic modification of cancer cells is able to induce an anticancer immune response. NOD.Cg-*B2m*^{tm1Unc}*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG^{TM}-b2m) mice are immunodeficient mice bearing an IL-2 receptor common γ-chain mutated gene together with a *Scid* mutation of the *Prkdc* gene (which encodes the catalytic subunit of a DNA-dependent protein kinase, preventing development of B and T cells) in the β2-microglobulin (MHC I)-deleted background (the absence of MHC class I molecules delays the GvHD). NSG^{TM}-b2m mice can be engrafted with human PBMCs to generate humanized NSG (Hu-PBMC NSG) mice with a functional human immune system. These mice can be used as a functional tool for cancer research, indeed patient tumor tissues can be engrafted into Hu-PBMC NSG mice. Our idea is to generate patient-derived xenografts (PDX) model by the implantation of fresh human colorectal tumor tissues into the humanized mice engrafted with PBMCs from the same patient from whom the PDX was derived, and evaluate the effect of compounds able to induce metabolic modification in tumor cells on the immune response. Specifically, we will generate Hu-PBMC NSG mice by transferring PBMCs and tumor tissues from patients with CMS3 CRC, thus obtaining humanized mice with the immune system and the PDX from the same patient. After transfer, mice will be divided in groups (5 mice/group) and each group will be treated with compounds, previously identified *in vitro*, able to correct the metabolic alteration characterizing the CMS3 tumor. Thus, we will proceed with the characterization of peripheral frequency, cell phenotype and function of different immune cell populations.

6. Further details on the overall methods that will be used in this project

This project will be a two-year effort involving different research groups with

complementary scientific background and expertise, each leader in the respective research field. Prof. Landriscina will be the Principal Investigator and the research group of the University of Foggia will be involved in studying the interplay between cell metabolism and gene expression/epigenetic reprogramming. Prof. Landriscina is also Principal Investigator of a research group at the IRCCS-CROB of Rionero in Vulture, Potenza. The IRCCS-CROB will provide: i) the tissue bank of human tumor specimens, ii) the genomic platform for RNA-seq and DNA methylation analysis by Illumina and Thermo Fisher. The group of Prof. Giannoni at the University of Florence will perform metabolomics studies. The group of Prof. Esposito at the Federico II University in Naples will provide the expertise to study the interplay between metabolic remodeling and post-transcriptional mechanisms of gene expression. The group of Prof. Matarese at the University Federico II of Naples will study the effect of tumor metabolic modulation on the immune response. Regarding immunological experiments proposed in Task 4 and 5, the following analyses will be performed *in vitro* and in mice:

1. Evaluation of cytokines production by immunoassays based on Luminex technology: GM-CSF, IFN alpha, IFN gamma, IL1α, IL1β, IL1RA, IL2, IL4, IL5, IL6, IL7, IL12p70, IL13, IL15, IL18, IL31, TNFα, TNFβ, BTLA, CD80, CD152 (CTLA4), CD27, CD28, CD137 (4-1BB), GITR, HVEM, IDO, LAG3, PD1, PD-L1, PD-L2, TIM3, Arginase1, CD73 (NT5E), CD96 (Tactile), E-Cadherin, Nectin2, MICA, MICB, PVR, Siglec7, Perforin, Siglec9, ULBP1, ULBP3, ULBP4.
2. PBMCs polychromatic flow cytometry immunophenotyping (staining for CD19, CD20, CD3, CD4, CD8, CD25, CD45RA, CD14, CD11b).
3. Analysis of frequency and phenotype of conventional T (Tconv) and regulatory T (Treg) cells. For this aim, we will evaluate the frequency of peripherally derived Treg (pTreg) cells (evaluated as $CD4^{+}Foxp3FoxP3^{+}CD25^{hi}CD127^{-}$) together with the expression of different Treg-specific differentiation/activation markers (such as CD39, CD69, CD71, CD127, CD25, PD-1, CTLA-4, GITR, CCR7 and CD62L), the proliferation marker (Ki67) and P-S6 phosphorylation (as readout of mTOR pathway activation).
4. To evaluate the activation status of T cells after co-culture of PBMCs with CMS1 and CMS3 cell lines, we will explore the activity of CD28-PI3K-AKT and TCR-ZAP70-RAS-ERK, the two major functional signaling pathways involved in T cell activation. Thus, we will also evaluate by western blot the expression of pAKT, AKT, pZAP70, ZAP70, pLCK, LCK, pERK1/2, ERK1/2, PI3K, pSTAT5 and STAT5 in PBMC co-cultured with tumor cell lines.

7. Work carried out and preliminary results

Our group is deeply involved in studying the crosstalk between cancer metabolism, epigenetic modifications and gene expression reprograming in human CRC with the aim to understand how cell metabolism modifies the tumor behavior at biological and clinical levels. In these studies, the mitochondrial HSP90 molecular chaperone, TRAP1 was modulated to modify the balance between oxidative and glycolytic metabolism and assess its consequence on gene expression/epigenetic reprograming. Indeed, TRAP1 is known to bind and inhibit succinate dehydrogenase and cytochrome c oxidase, respectively complexes II and IV of the respiratory chain, and, thus, is responsible for the downregulation of oxidative phosphorylation and the upregulation of glycolytic metabolism by enhancing the activity of phosphofructokinase-1 (11). DNA methylation and gene expression analysis were comparatively analyzed in high oxidative phosphorylation (TRAP1 KD cells) vs high glycolytic activity cell (TRAP1-expressing cells) systems. In TRAP1-knockdown (KD) HCT116, we identified 68 hypermethylated and downregulated and 55 hypomethylated and upregulated genes. In addition, TRAP1 KD HCT116 cells are characterized by a parallel increase of total methylated cysteine levels and D-2-hydroxyglutarate intracellular levels (Figure 1).

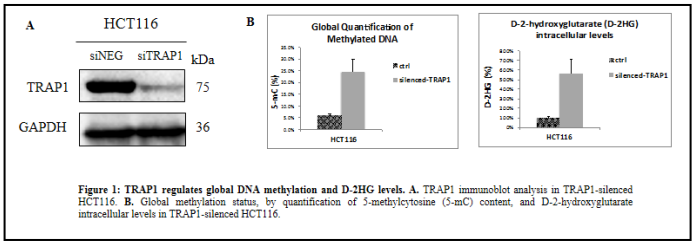

Figure 1: TRAP1 regulates global DNA methylation and D-2HG levels. A. TRAP1 immunoblot analysis in TRAP1-silenced HCT116. **B.** Global methylation status, by quantification of 5-methylcytosine (5-mC) content, and D-2-hydroxyglutarate intracellular levels in TRAP1-silenced HCT116.

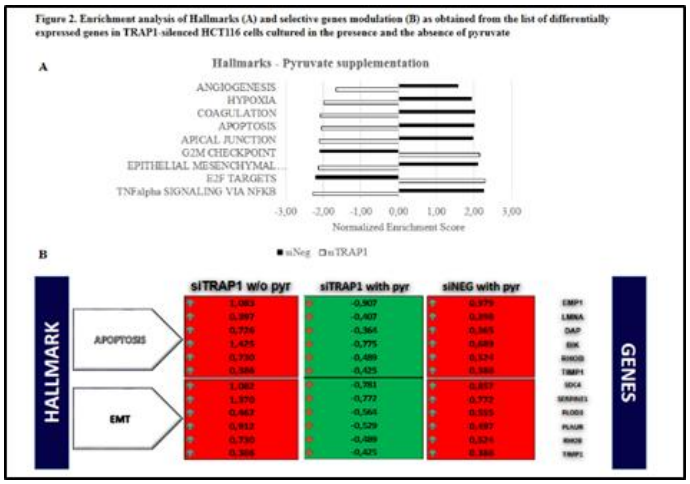

Figure 2. Enrichment analysis of Hallmarks (A) and selective genes modulation (B) as obtained from the list of differentially expressed genes in TRAP1-silenced HCT116 cells cultured in the presence and the absence of pyruvate

Gene expression profiling was also performed in TRAP1 KD HCT116 cells cultured with or without pyruvate supplementation showing that the remodeling of the balance between mitochondrial respiration and glycolysis elicited by pyruvate results in a significant modification of gene expression profile. Hallmark analysis showed that pyruvate supplementation induces a strong gene expression reprogramming that depends on TRAP1 expression: specific hallmarks and selective representative genes are activated/inhibited by pyruvate supplementation depending on TRAP1 background. These data suggest a mechanism of gene expression regulation that depends on the metabolic state of cancer cells (Figure 2). Our research group is also involved in the characterization of novel prognostic/predictive biomarkers in human CRC. Recently, epigenetic modifications were suggested as potential tools to predict patients' outcome in clinics. To this purpose, we performed a global DNA methylation profiling on a cohort of human primary-resistant and sensitive mCRCs and this was analyzed respect to main clinical parameters. An epigenetic signature of eight hypermethylated genes was characterized, able to identify mCRCs with poor prognosis, CIMP-high and MSI-like phenotype (12). The MSI-like/CIMP-high cohort in comparison with the MSS/CIMP-low cohort will be used in this project for the validation of gene expression/epigenetic data.

8. Expected results and relevant corresponding milestones

This project will investigate the paradigm that metabolic alterations sustain several hallmarks of cancer, this representing the rational to point at metabolism as a very interesting and novel therapeutic target. Objective of the present project is to characterize molecular mechanisms responsible for metabolic rewiring in human CRC and establish a mechanistic interplay and a hierarchical organization between cancer metabolic remodeling, gene expression reprogramming and immune escape mechanisms. More specifically, the project will prove whether and how metabolic pathways can be targeted to improve the activity of ICIs in MSS mCRCs, this representing an unsolved issues in this field. As this is a recent and rapidly evolving field, with several aspects often controversial, the present research proposal starts from solid preliminary data, as a prerequisite to provide future perspectives for novel strategies to disrupt networks of integrated control in cancer cells. In such a context, preclinical and clinical evidence suggests that metabolism-targeting drugs can efficiently interfere with the activity of immune therapies. New therapeutic approaches combining metabolic drugs with ICIs need to be designed, with the aim to overcome immune tolerance. Even though few specific drugs are currently available, it is expected that their number will increase, opening new therapeutic perspectives.

Relevant milestones are:

1. The biological characterization of metabolic, gene expression and epigenetic differences between CMS1 and CMS3 tumor models;
2. The characterization of a direct relationship between metabolic, gene expression and epigenetic alterations and immune tolerance mechanisms;
3. The validation of the hypothesis that the targeting of metabolic/epigenetic pathway

represents a strategy to revert immune escape mechanisms.

	Year 1				Year 2			
Timeline (month end)	**3**	**6**	**9**	**12**	**3**	**6**	**9**	**12**
Task 1								
Task 2								
Task 3								
Task 4								
Task 5								

9. References and relevant publications by the research group, already available

1) André T, *et al*. Pembrolizumab in Microsatellite-Instability-High Advanced Colorectal Cancer; KEYNOTE-177 Investigators. N Engl J Med. (2020);383(23):2207-2218.
2) Guinney J, *et al*. The consensus molecular subtypes of colorectal cancer. Nat Med. (2015); 21(11):1350-6.
3) Cerezo M, *et al*. Cancer cell metabolic reprogramming: a keystone for the response to immunotherapy. Cell Death and Disease (2020) 11:964.
4) Reid MA, *et al*. The impact of cellular metabolism on chromatin dynamics and epigenetics. Nat Cell Biol (2017);19(11):1298-1306.
5) Zhang D, *et al*. Metabolic regulation of gene expression by histone lactylation. Nature (2019);574 (7779):575-580.
6) Xiao M, *et al*. Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes Dev (2012); 26:1326-38
7) Eastman G, *et al*. Following Ribosome Footprints to Understand Translation at a Genome Wide Level. Comput and Struct Biotechnol J. (2018); 16:167–176
8) Sciacovelli M *et al.* Fumarate is an epigenetic modifier that elicits epithelial-to-mesenchymal transition. Nature. (2016); 537(7621): 544–547.
9) Carbonneau M, *et al*. The oncometabolite 2-hydroxyglutarate activates the mTOR signalling pathway. Nat Commun. (2016); 7:12700.doi: 10.1038/ncomms12700.
10) Chang CH, *et al*. Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell. (2013); 153:1239–1251.
11) Maddalena F, Condelli V, Matassa DS, Pacelli C, Scrima R, Lettini G, Li Bergolis V, Pietrafesa M, Crispo F, Piscazzi A, Storto G, Capitanio N, Esposito F, Landriscina M. TRAP1 enhances Warburg metabolism through modulation of PFK1 expression/activity and favors resistance to EGFR inhibitors in human colorectal carcinomas. Mol Oncol. (2020); 14(12):3030-3047.
12) Condelli V, Calice G, Cassano A, Basso M, Rodriquenz MG, Zupa A, Maddalena F, Crispo F, Pietrafesa M, Aieta M, Sgambato A, Tortora G, Zoppoli P, Landriscina M. Novel Epigenetic Eight-Gene Signature Predictive of Poor Prognosis and MSI-Like Phenotype in Human Metastatic Colorectal Carcinomas. Cancers (2021), 13, 158.

PERSONNEL INVOLVED IN THE RESEARCH

Name and date of birth	Role on Project	Fellowship required	Effort on project (%)	Present position
Prof. Matteo Landriscina (24/10/1968)	Principal Investigator	No	30%	Full Professor of Medical Oncology, Department of Medical and Surgical Sciences, University of Foggia
Dr. Giuseppina Bruno (09/01/1988)	Researcher	No	40%	Assistant Professor of Medical Oncology,

				Department of Medical and Surgical Sciences, University of Foggia
Dr. Guido Giordano (25/05/1981)	Researcher/Medical Oncologist	No	40%	Assistant Professor of Medical Oncology, Department of Medical and Surgical Sciences, University of Foggia
Dr. Annamaria Piscazzi (23/12/1966)	Researcher	Yes	70%	Research Fellow, Department of Medical and Surgical Sciences, University of Foggia
Dr. Valentina Condelli (05/08/1982)	Researcher	No	40%	Researcher at IRCCS-CROB of Rionero in Vulture
Dr. Fabiana Crispo (08/05/1982)	Researcher	No	40%	Post-doc at IRCCS-CROB of Rionero in Vulture
Dr. Michele Pietrafesa (12/10/1986)	Researcher	No	40%	Researcher at IRCCS-CROB of Rionero in Vulture
Dr. Pietro Zoppoli (08/05/1977)	Researcher	No	20%	Researcher at IRCCS-CROB of Rionero in Vulture
Prof. Franca Esposito (30/06/1955)	External Collaborator	No	30%	Full Professor of Biochemistry, Department of Molecular Medicine and Medical Biotechnology, University of Naples “Federico II”
Prof.Giuseppe Matarese (23/08/1970)	External Collaborator	No	30%	Full Professor of Immunology and General Pathology, Department of Molecular Medicine and Medical

				Biotechnology, University of Naples “Federico II”
Prof. Elisa Giannoni (01/12/1973)	External Collaborator	No	30%	Associated Professor of Biochemistry, Department of Experimental and Clinical Biomedical Sciences, University of Florence

DESCRIPTION OF THE WORK FOR EVERY UNIT OF PERSONNEL

1. **Prof. Matteo Landriscina**: Principal Investigator.
   Coordination of the project and publication of final study results in international scientific journals.

2. **Dr. Giuseppina Bruno:** Research
   DNA methylation profiling of CMS1 and CMS3 cell lines to identify genes potentially methylated/downregulated through differential accumulation of metabolites identified in Task 1 (**Task 2**). Validation of multiomic data using metabolites and metabolic/epigenetic inhibitors selected from Task1 and 2 (**Task 3**).

3. **Dr. Guido Giordano:** Research/Medical Oncologist
   Enrollment of colorectal cancer patients, collection of tumor samples and clinical data. (**Tasks 2, 3 and 5**).

4. **Dr. Annamaria Piscazzi:** Research
   RNA-sequencing in CMS3 cells compared to CMS1, also matching mRNA expression levels to the corresponding methylation status (**Task 2**). Immunohistochemistry, western blot and RT-qPCR validating analyses in human CMS1 and CMS3 CRCs and ELISA assay on plasma from the same patients (**Task 2**). Validation of multiomic data using metabolites and metabolic/epigenetic inhibitors selected from Task1 and 2 (**Task 3**).

5. **Dr. Valentina Condelli:** Research
   MSI-like/CIMP high cohort vs MSS/CIMP-low cohort (already profiled for DNA methylation) analyses for CMS subtypes using public datasets of human CRCs (**Task 2**). Validation of multiomic data using metabolites and metabolic/epigenetic inhibitors selected from Task1 and 2 (**Task 3**).

6. **Dr. Fabiana Crispo:** Research
   DNA methylation profiling CMS1 and CMS3 cell lines to identify genes potentially methylated/downregulated through differential accumulation of metabolites identified in Task 1 (**Task 2**). Validation of multiomic data using metabolites and metabolic/epigenetic inhibitors selected from Task1 and 2 (**Task 3**).

7. **Dr. Michele Pietrafesa:** Research
   MSI-like/CIMP high cohort vs MSS/CIMP-low cohort (already profiled for DNA

methylation) analyses for CMS subtypes using public datasets of human CRCs (**Task 2**). Validation of multiomic data using metabolites and metabolic/epigenetic inhibitors selected from Tasks1 and 2 (**Task 3**).

8. **Dr. Pietro Zoppoli**: Researcher/Bioinformatician.
   Statistical analysis of multiomic data (**Task 2**).

9. **Prof. Franca Esposito:** External Collaborator.
   Coordination of the research group at the Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", that will be in charge of ribosome profiling experiments (**Task 2**).
   Esposito's collaboration letter is attached as supplementary document 2.

10. **Prof. Giuseppe Matarese:** External Collaborator.
    Coordination of the research group at the Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", that will be in charge of immunological experiments (**Tasks 4 and 5**). More specifically, they will perform co-cultures of CMS1 and CMS3 cell lines with PBMCs and cell supernatant assay to evaluate cytokines and other soluble proteins involved in antitumor immune response; PBMCs FACS polychromatic flow cytometry and transwell migration assays (**Task 4**). Hu-PBMC NSG mice generation and subcutaneously implant of tumor tissues (**Task 5**).
    Matarese's collaboration letter is attached as supplementary document 3.

11. **Prof. Elisa Giannoni:** External Collaborator.
    Coordination of the research group at the Department of Experimental and Clinical Biomedical Sciences, University of Florence, that will be in charge of metabolomics experiments (**Task 1**). More specifically they will perform metabolomic analyses of CMS1 *vs* CMS3 CRC cells by: Seahorse analysis, high-resolution respirometry, biochemical assays, steady-state GC- and LC-mass spectrometry analysis of metabolome and exometabolome (**Task 1**).
    Giannoni's collaboration letter is attached as supplementary document 4.

Budget Form/2 years

1.	research costs	70.000
2.	Instruments	
3.	Indirect costs	10.000
4.	**Sub-total**	**80.000**
5.	Overheads 10%	8.000
6.	Fellowships	32.000
7.	**Total**	120.000

Justifications
Itemized research costs:

- Tissue culture reagents for cell lines (media, sera, selection antibiotics, growth factors, plasmids, transfection kits, siRNAs) 5.000 €
- Protein biochemistry reagents (chemicals, western blot, immunofluorescence and immunohistochemistry reagents, primary and secondary antibodies, reagent for flow-cytometry) 5.000 €
- Molecular biology reagents (plasmids, semiquantitative and real time RT-PCR reagents, DNA and RNA extraction kits for mammalian cells, enzymes, DNA extraction kits for bacteria). 10.000 €
- Chips and other reagents for multiomic analysis. 20.000 €

- Reagents for metabolomic analysis. 10.000 €
- Animal experimentation. 20.000 €
- Indirect costs: Instrument maintenance, cost for publication in international journals, meetings 10.000 €
- Fellowship: one contract for 18 months for a biologist with expertise in the field. 32.000 €

EXISTING/PENDING SUPPORT: no existing support is at present available for this project. A partially overlapping research proposal has been submitted to the PRIN 2019 grant application of the Italian Ministery of Scientific Research.

SUGGESTED REVIEWERS (MAX 3)

1. Prof. Igor Prudovsky, Maine Medical Center Research Institute, Portland, Maine, USA, prudoi@mmc.org;
2. Prof. Alessandra Cassano, Associate Professor of Medical Oncology, Catholic University, Rome, alessandra.cassano@unicatt.it
3. Prof. Mario Scartozzi, Full Professor of Medical Oncology, University of Cagliari, marioscartozzi@unica.it

BIOETHICAL REQUIREMENT

1. Human experimentation: YES, use of human specimens. The informed consent form approved by the Ethical Committee is attached as addendum A

2. Animal experimentation: YES – addendum B is attached

Declaration

I shall confirm to the Declaration of Helsinki in its latest version.
I shall also apply the Bioethics Convention of the Council of Europe.
In implementing the proposed research, I shall adhere most strictly to all existing ethical and safety provisions applicable.
Before start of the research, I shall obtain clearance from the competent ethical committee in case of involvement of human subjects in the research and /or in case of other ethical implications.
I shall conform with all regulations protecting the animals used for research purpose.

Date: 14/01/2021 Name of PI: Prof. Matteo Landriscina

LANDRISCINA MATTEO 14.01.2021 18:51:55 UTC

Principal investigator's signature: Prof. Matteo Landriscina

Authorized Administrative Official's signature: Prof. Pierpaolo Limone

Foggia, 14/01/2021

Si autorizza al trattamento dei dati ai sensi dell'articolo 5 del Regolamento (UE) 2016/679

LANDRISCINA MATTEO 14.01.2021 18:52:58 UTC

Firmato digitalmente da:
LIMONE PIERPAOLO
Firmato il 15/01/2021 13:00
Seriale Certificato: 805571420980313619458989276191058648 44
Valido dal 15/11/2019 al 14/11/2022
ArubaPEC S.p.A. NG CA 3