Fondazione AIRC

Proposal submitted in response to the Call:

MFAG 2020

YEAR 2020

FULL SUBMISSION

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TITLE PAGE

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Proposal Title
Insight into TGFb/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy

Type of grant	MFAG 2020

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Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy.

1. Summary statement

Although immune checkpoint blockade (ICB) therapies generated great hope in oncology, their efficacy is still limited to ~15-25% of cancer patients[1]. Accurate understanding of the biological mechanisms underlying ICB response is an unmet need and lack of knowledge prevents the development of new combinatorial therapies with increased clinical efficacy. The tumor microenvironment (TME) is implicated in the modulation of the antitumor response[2], however the contribution of the vasculature still remains elusive. Here, ***we will elucidate the role of tumor associated endothelial cells (TECs) in melanoma immune evasion and ICB resistance.*** Several factors will contribute to successfully carry out this proposal: my multi-disciplinary international scientific experience, leadership skills and commitment to cancer research as demonstrated by my recent last-author work[3]; an outstanding collaborator network; immediate availability of melanoma samples as well as unique *in vivo* models to conduct mechanistic studies.

2. Background

Despite positive clinical outcomes of immunotherapies, often melanoma develops primary and acquired resistance[4]. Our understanding of the biological mechanisms underlying ICB response in melanoma is insufficient to develop strategies to overcome resistance. Recently, the TME attracted great attention as a major determinant of immune evasion[2]. Among the TME cellular components, TECs contribute to tumor progression in several ways. First, TECs undergoing angiogenesis support melanoma development[5]. Second, endothelial cells experience anergy, a cellular process driven by proliferation stimuli highly abundant in the TME, such as VEGF, which prevents endothelial activation and reduces immune cell recruitment to the site of inflammation[5], generating a real tumor endothelial barrier. Third, TECs can originate cancer-associated fibroblasts (CAFs) through endothelial-mesenchymal transition (EndMT), a dedifferentiation process induced by high TGFβ levels in the TME[6]. Although several data describe how TECs undergo angiogenesis, very little is known about the role of TEC anergy and EndMT in melanoma. Here, **we hypothesize that both TEC anergy and EndMT are main determinants of melanoma resistance to immunotherapy.** Supporting our hypothesis: *i)* ICB non-responder patients almost invariantly present poor immune cell infiltration into the tumor, which we believe is the result of a decreased interaction between the leukocytes and the vessel walls[5]; and *ii)* ~40% of CAFs into a tumor derives from EndMT[6]. Synthetically active CAFs support tumor growth, metastasis dissemination and extracellular matrix deposition contributing to immune escape and therapy resistance[6]. Notably, the expression levels of the EndMT master gene Zeb1 correlate with poor prognosis and resistance in BRAF-mutant melanoma[7], corroborating the crucial role of EndMT in melanoma development. Moreover, TGFβ-induced Zeb1 harnesses melanoma cell de-differentiation favoring plasticity and drug resistance[7]. Here, we aim to shed light on the largely unexplored TEC contribution to melanoma ICB resistance focusing on TGFβ/Zeb1 circuitry and its impact on tumor endothelial barrier generation.

3. Work program (WP)

The present proposal aims to provide an answer to the following key questions: 1) *Do TECs contribute to melanoma resistance to BRAF inhibitor (BRAFi) and ICB therapy?* 2) *Is the TGFβ/Zeb1 circuitry involved in TEC-driven stromal remodeling and immune escape?* 3) *Are small molecules interfering with TGFβ/Zeb1 circuitry able to prevent/delay melanoma resistance to BRAFi and ICB therapy?*

WP 1. Assessment of TEC contribution to melanoma resistance.

Task 1: TEC *ex vivo* analysis. TEC contributive role to immune evasion will be explored performing a retrospective analysis on a cohort already available of 30-40 melanoma patients (stratified by disease stage and follow-up data). Concurrently, Dr Bussolino (University of Turin) will provide already collected melanoma samples derived from mice bearing $BRAF^{V600E}$ melanoma, the most frequent genetic alteration in melanoma, treated with Vemurafenib (BRAF inhibitor), anti-PD1 and Vemurafenib+anti-PD1 (COMBO) at different time points (**Fig.1**). Immuno-staining (IS) will show: a) immune infiltration (analyses of lymphoid (T, B and NK cells)

and myeloid (macrophages, granulocytes and dendritic cells) biomarkers); b) TEC anergy (evaluation of molecules involved in leukocyte recruitment: selectins, integrins and chemokines); c) EndMT (analysis of endothelial/mesenchymal/fibrotic markers and involved transcription factors including Zeb1, **Fig.1**). These studies will provide: a) correlations among TEC anergy/EndMT/immune response and melanoma survival/stage/recurrence and b) insight into the status of TEC activation/dedifferentiation in melanoma.

Task 2: TEC *in vivo* analysis. To follow TEC anergy and EndMT *in vivo*, we will generate conditional endothelial reporter mice (iEC^{tomato}) mating endothelial inducible mice (*VECad-CreERT2* mice) with reporter mice (*R26Rosa-lox-Stop-lox-tdTomato-hrLuc* mice; both already available in the lab). iEC^{tomato} mice will be subcutaneously (sc) injected with syngenic BRAF^{V600E} melanoma cells and treated as described in Task 1 after 2-4 weeks tumor reached a volume of 250-300 mm^3. Tumor outgrowth and response to therapy will be monitored bi-weekly. Fresh melanoma samples will be collected at different time points, analyzed to gain insight into secretome (using antibody arrays) and processed to isolate and characterize TECs (RNASeq). These studies will inform on melanoma secreted molecule and TEC plasticity contribution to immune evasion.

WP2. Targeting of TGFβ/Zeb1 circuitry to overcome TEC-driven melanoma resistance.

Task 1: TGFβ/Zeb1 *in vitro* analysis. As the TGFβ/Zeb1 circuitry supports cell dedifferentiation and metastasis dissemination[8], we aim to target it for cancer therapy. Initially, to dissect the molecular mechanisms of TEC anergy and EndMT, we will take advantage of an *in vitro* model already established by the PI, where primary endothelial cells (HMVECs) are treated for 5 days with TGFβ to induce EndMT. TEC anergy and EndMT will be evaluated by RNASeq; TGFβ-dependent Zeb1 DNA binding by ChIPSeq; and functional assays of transendothelial migration. Techniques successfully used by the PI in the past[9,10]. This task will provide novel targets to revert TEC phenotype that can be exploited in the subsequent tasks to favor ICB response.

Task 2: Analysis of Zeb1 knockout (KO) in TECs. To further investigate Zeb1 role in TECs, we will generate an inducible endothelial Zeb1 KO mouse (Zeb1$^{iEC-/-}$) in collaboration with the Transgenic mouse facility-IBBC-CNR (Dr Chiani and Dr Gambadoro). Zeb1$^{iEC-/-}$ mice will be sc injected with BRAF^{V600E} melanoma cells and treated as described in Aim1/Task1. Tumor outgrowth and response to therapy will be monitored bi-weekly. We expect that Zeb1 KO in the endothelium will favor melanoma ICB response. TEC anergy and EndMT markers will be evaluated by IS and qRT-PCR with a particular emphasis on targets identified in Aim2/Task1.

Task 3: Pharmacological targeting of TGFβ/Zeb1 circuitry. To translate our studies to the clinic, we will evaluate the therapeutic efficacy of small molecules interfering with TGFβ/Zeb1 circuitry in combination with COMBO, using iEC^{tomato} mice bearing BRAF^{V600E} melanoma. The following small molecules will be combined with COMBO: TGFβ inhibitors (galunisertib and fresolimumab); Zeb1 indirect inhibitor (AA6 a small molecule decreasing Zeb1 levels[3]); class I HDAC inhibitors (MS275, SAHA and FK228), for the cooperation between class I HDACs and Zeb1 in transcriptional repressor complexes[9]. Tumor outgrowth and response to therapy will be monitored bi-weekly. These studies will provide a rationale to move our discoveries to the clinic.

4. Potential impact on the oncology field

We will implement specific dissemination strategies oriented towards clinicians dealing with melanoma patients experiencing primary and acquired ICB resistance. We anticipate that this study will have a direct impact on the taxonomy of melanoma with optimization of patient stratification and treatment, opening the avenues to new combinatorial therapies aimed to increase ICB efficacy in melanoma patients.

PI QUALIFICATION

Background, expertise and significant experience abroad. Since 2005, I am actively working in the field of physiopathology, mostly focusing on translational medicine to elucidate new molecular mechanisms involved in human disease. I accumulated vast experience on the vascular function performing experiments on primary endothelial cells and endothelial precursors. Most of my previous studies have been conducted exploiting *ex vivo*, *in vivo* and *in vitro* analyses. I started to deal with the transcriptional repressor Zeb1 during my PhD in

Immunological Sciences branching out my studies to different contexts. Specifically, I dissected Zeb1 role in human endothelial cells exposed to oxidative stress, in an antineoplastic-associated cardiotoxicity mouse model, during differentiation of mouse embryonic stem cells[9] and in a mouse model of breast-associated cancer metastasis[3]. I acquired a strong background on Zeb1 biology and developed several tools to pursue my studies. I spent 5 years (2012-2016) at the Division of Cardiovascular Epigenetics-Goethe University (Frankfurt am Main, Germany) under Dr. Gaetano supervision. There, I broadened my scientific horizons to epigenetics, contributing to the screening of novel compounds active on cell epigenetic landscape. Further, I became confident with OMICs, including RNASeq and ChIPSeq, being able to profitably interact with bioinformaticians. Thus, this proposal perfectly fits with my expertise and fulfils the MFAG call.

Commitment to cancer. My constant interest in Zeb1 functions prompted me to ask for the question of whether this master transcriptional regulator can be targeted for cancer therapy. Recently, I demonstrated Zeb1 role in stemness maintenance[9] and I will apply this knowledge to the de-differentiation mechanisms occurring in the TME. My first last author work permitted me to start broadening my interests to the cancer field exploiting my experience on Zeb1 and epigenetics[3]. I do believe that cancer represents the perfect field to further investigate Zeb1 particularly focusing on its role in the TME. Zeb1 has been already characterized for its role in cancer cell plasticity, but its contribution to TEC dependent immune evasion has not been analyzed to date. I am excited to strengthen my commitment to cancer research exploiting my past background while setting up my laboratory as an independent investigator.

Qualifications and motivations to become independent and acquire leadership. Since I obtained a permanent position as researcher at CNR (December 2016), I am actively seeking funds for my research projects and to establish my lab. I already mentored and coordinated 3 PhD students, 2 Technicians and 2 Erasmus students when I was in Germany. It was an enjoyable experience. There is no bigger satisfaction to realize that your students are scientifically and technically growing and achieving significant career goals also because of your guidance and teaching. All my PhD students already published at least one first author paper under my supervision and obtained their doctoral degree. A technician is working on a clinical trial at Goethe University.

Former supervisor interaction. My former supervisor research focuses on aging and cardiovascular disease. Dr. Gaetano is not actively pursuing the research themes described in this proposal and no overlap is present.

The AIRC support will complete my transition to independence and permit my group to pursue interesting questions in the cancer field, where my expertise on endothelium, Zeb1 and epigenetics can be applied. I do believe that this proposal will fulfill the knowledge gap on an overlooked biological process, the role of TECs, which promises being extremely important in ICB resistance. The molecular focus on TGFβ/Zeb1 circuitry will provide new therapeutic targets that will facilitate to overcome melanoma ICB resistance. Indeed, we expect to identify novel druggable targets to prevent TEC anergy and EndMT responsible of immune evasion.

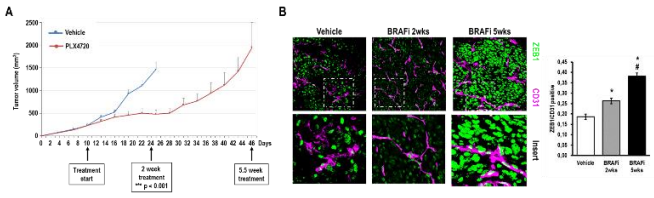


Fig. 1 In vivo effects of PLX4720 on tumor growth and on Zeb1 expression in melanoma endothelium. A) Tumor growth of mice bearing D4M cells treated with vehicle (n=7; blue line) or PLX4720 (n=7; red line). **B)** Left: Representative images, related insets and quantification of melanoma tissues treated with PLX4720 probed with anti-CD31 (magenta) and anti-Zeb1 (green). Vehicle used as solvent control. Magnification 40X.

References: **1.** Ribas A, Wolchok JD *Science* **359**:1350 (2018). **2.** Klemm, F and Joyce, JA *Trends Cell Biol* **25**: 198 (2015). **3.** Atlante, S et al *Cell Death Dis* **9**: 756 (2018). **4.** Gide, TN et al *Clin Cancer Res* **24**:1260 (2018). **5.** Georganaki, M. et al *Front Immunol* **9**: 3081 (2018). **6.** Medici A and Kalluri R *Semin Cancer Biol* **22**: 379 (2012). **7.** Richard, G et al *EMBO Mol Med* **8**: 1143 (2016). **8.** Lee, SY et al *Mol Cancer* **16**: 10 (2017). **9.** Cencioni, C et al *Nat Commun* **9**: 1281 (2018). **10.** Cencioni, C et al *Eur Heart J.* **34**:2007 (2013).

Consiglio Nazionale delle Ricerche
Istituto di Analisi dei Sistemi ed Informatica "Antonio Ruberti"

Institutional commitments

Ref: 2020 My First AIRC Grant (MFAG) - Call for applications

***Applicant**:* Dr Chiara Cencioni

Title of the application: Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy.

26/02/2020

In my position as Head of the Hosting Institution, the Istituto di Analisi dei Sistemi ed Informatica "Antonio Ruberti" (IASI) (Institute for Systems Analysis and Computer Science), it is my great pleasure to write this letter in support of Dr. Chiara Cencioni to carry out her research proposal as an independent principal investigator (PI).
IASI is an institute of the Italian National Research Council (CNR) established in 2002 from a partnership among CNR scientists with complementary expertise and interests derived from two Institutes: the Institute for Systems Analysis and Computer Science and the Centre for the Study of the Pathophysiology of Shock, both established in 1969. The main institute site is located in via dei Taurini, 19 -- Rome (Italy), where mathematical modelling studies for biomedicine and in particular for tumor growth are conducted together with bioinformatics analyses of genes involved in tumor onset. The secondary institute site in Rome is dislocated into two units at Policlinico Gemelli and at the Medical School of Catholic University of Sacred Heart (UCSC), which research activities focus on physiopathology, metabolism, oncology, and immunology to uncover novel molecular and cellular mechanisms contributing to the development of human pathologies. IASI currently includes about 50 permanent staff members (scientists, technicians, and administrative personnel) plus a cohort of post-doctoral scientists, PhD students and associated members affiliated with universities in Rome, Florence, L'Aquila, Catania, Pisa, Montreal, working together. The secondary IASI site at UCSC represents the perfect environment where to conduct Dr. Cencioni´s project proposal for the presence of several researchers already funded by AIRC and of all the facilities necessary to achieve the goals described in the research plan.

Percentage of time dedicated to the project

The applicant will have at least 50% of the time dedicated to the MFAG research project.

Lab and office space

Dr. Cencioni is a tenured junior CNR researcher since December 2016, who joined IASI in September 2019 after the suppression of the CNR Institute of Cell Biology and Neurobiology (IBCN). She works at the secondary IASI site at the Institute of Medical Pathology and Human Physiology belonging to the Medical School of UCSC, as regulated by the agreement signed between IASI and UCSC in January 2020. She has been granted already with the necessary infrastructures to develop her research projects, including free access to all facilities available at UCSC (see below). She has been successfully integrated in this cooperative environment and set

up a collaboration with the Department of Oncology of the University of Turin to obtain melanoma samples and with the Transgenic Animals Facility of CNR in the Monterotondo campus, in the recently established CNR Institute of Biochemistry and Cell Biology (IBBC).

Hosting Institution facilities and resources

Due to the strict interconnection among all the above-mentioned research centres, the applicant will benefit from training opportunities organized by all these Institutions. The PI will have the opportunity for critical professional interactions with senior colleagues already working in the field of molecular oncology and will benefit from outstanding invited speakers giving seminars at UCSC and at IASI. The laboratories located there are fully equipped for standard molecular/cell biology experiments. Thanks to the agreement between IASI and UCSC, the applicant will have free access also to the following equipment: QS Series Real-Time PCR (Applied Biosystems); QX-200 Droplet Digital PCR (Bio-Rad); Bioruptor Sonicator; Nanodrop; Confocal microscope; reverted-phase contrast-, immunofluorescence-, video time-lapse-, stereotactic-microscopes, flow cytometer, thermocycler, Instant Imager, VersaDoc 3000, Victor2 (fluorometer, luminometer, ELISA), ELISA Readers/Washers, Seahorse Biosciences® XP technology, spectro-photometer, cryostat, microtome, vibratome; cold room; P2 facility for virus work; and a recently renovated animal facility.

Authorship in publications

In all publications stemming from the research carried out with this grant, the applicant will be last author and corresponding author as well.

PI scientific independence

The unit headed by Dr Cencioni will be indicated as an independent unit in IASI staff directories, website and public reports. I will provide full commitment to assist the PI during her “transition to independence”, supporting all necessary steps to reach a position of scientific independence within IASI by the end of the grant.

In conclusion, I fully support Dr Cencioni’s application to MFAG call 2020. This will be of great value for IASI and of high importance for her future scientific career. She will consolidate her background in Zeb1, epigenetics and metabolism translating it to molecular oncology. MFAG will offer a great opportunity to young scientists like Dr Cencioni to develop their own ideas and be immediately competitive even in a field of study where competition is undoubtedly fierce. I do believe that AIRC support will advance her career as a fully independent cancer researcher.

Signature

This document does not require a signature. By signing the application, Legal Representative of the Hosting Institution certifies that he/she will comply with the conditions described in this letter in order to foster the applicant’s research career and his/her independence.

ABSTRACT

Background

The prevalence of BRAF mutations and the high immunogenicity of melanoma opened the avenue to tailored therapies based on BRAF inhibitors (BRAFi) and immune checkpoint blockers (ICB). These therapeutic approaches significantly improved progression-free survival of melanoma patients, but their efficacy is still limited because of widely variable responses and onset of primary and acquired resistance. Tumor associated endothelial cells (TECs) support tumor progression by angiogenesis, anergy and endothelial-to-mesenchymal transition (EndMT). TEC anergy and EndMT contribute respectively to immune evasion and stromal remodelling, crucial mechanisms for the development of melanoma resistance to BRAFi and ICB. Transforming growth factor β (TGFβ), which is increased by BRAFi treatment, alters vascular functions, induces mesenchymal transition activating the transcription factor Zeb1, inhibits immune surveillance ultimately establishing a tumor microenvironment supporting cancer growth and therapy evasion.

Hypothesis

The hypothesis is that melanoma resistance to BRAFi and ICB might develop as consequence of an impairment of TECs. Specifically, the abundance of proliferative stimuli and of TGFβ levels in the melanoma microenvironment could: 1) prevent immune cell recruitment inducing TEC anergy; and 2) contribute to stromal remodelling promoting EndMT. Since TGFβ/Zeb1 axis harnesses both mechanisms crucial for melanoma progression and resistance, it is possible to speculate that the combination of BRAFi and ICB with small molecules interfering with TGFβ/Zeb1 circuitry delay/prevent the onset of melanoma resistance rescuing TEC functions.

Aims

The proposal aims are: 1) description of responder and resistant melanoma immunophenotype; 2) assessment of TEC features supporting stromal remodeling, immune evasion and melanoma progression; 3) evaluation of TGFβ/Zeb1 circuitry involvement in TEC-driven melanoma resistance; 4) dissection of the role of Zeb1 in TEC function by establishing an inducible endothelial Zeb1 knockout mouse; 5) exploitation of a polypharmacology approach to overcome TEC-driven melanoma resistance based on TGFβ/Zeb1 circuitry targeting.

Experimental Design

These objectives will be pursued through a focus approach consisting in: 1) specific ex vivo analyses of human and mouse melanoma samples derived from responders and non-responders to BRAFi and ICB; 2) in vivo evaluation of TEC functions exploiting an endothelial reporter mouse model bearing BRAF mutant melanoma upon treatment with BRAFi, Anti-PD1 or both in combination; 3) cellular and molecular assays dissecting TGFβ/Zeb1 circuitry role in TEC anergy and EndMT; 4) generation of a conditional endothelial Zeb1 knockout mouse; 5) pharmacological specific targeting of the TGFβ/Zeb1 circuitry in combination with BRAFi and ICB and evaluation of melanoma resistance onset timing.

Expected Results

This proposal will increase the knowledge on the role of TEC in the onset of melanoma resistance and will shed light on main molecular determinants exploitable in novel combinatorial anti-neoplastic approaches enhancing BRAFi and ICB response in melanoma patients.

Impact On Cancer

This study will provide novel insights into pathways involved in TEC-driven melanoma resistance to BRAFi and ICB, fulfilling a knowledge gap on an overlooked biological process related to the role of TECs. The scientific framework promises to design attractive and feasible combinatorial therapeutic strategies aimed at increasing the clinical efficacy of BRAFi and ICB preventing/delaying melanoma resistance with an impact on the clinical management of melanoma patients.

Cencioni_Point-by point letter MFAG 2020.

The PI thanks the reviewers for their valuable comments and suggestions. Due to space restrictions, PI unified point-by-point response to reviewers expressing similar concerns. All changes in the revised version of the project proposal are tracked in red.

Q1 Reviewer #1 and #2: Focus on TGFβ/Zeb1 circuitry and related supporting preliminary data

A1: The present proposal aims to dissect the contribution of the endothelium-driven resistance to anti-cancer therapy through attenuation of immune cells recruitment and modulation of tumor microenvironment. Focus on Zeb1 transcription factor is supported by evidences in literature indicating an increase of its expression and activity in highly aggressive melanoma with an immunosuppressive profile (Most recent publications: PMID: 32503808; PMID: 31817719; PMID: 31388313). Interestingly, Zeb1 has also been described involved in MAPK inhibitor resistance in melanoma, driving drug adaptation (PMID: 27596438). Moreover, Zeb1 is a well-known driver of EndMT and has been described able to transcriptionally control IL-2 expression, the key cytokine for T cell functions (PMID: 19181930). Nevertheless, at present no investigation described Zeb1 role in endothelial-mediated resistance to anti-cancer therapy. According to preliminary data, Zeb1 expression increases in the nuclei of endothelial cells of PLX4720-treated mice bearing BRAF mutated melanoma showing signs of acquired resistance. Specifically, following the initial delay in melanoma outgrowth (after first 2 weeks of treatment), melanoma gradually started reaching a tumor volume comparable to vehicle group (no treatment) around 4-5 weeks showing melanoma drug adaptation (Fig. 1A). Of note, differently from what observed in whole melanoma tissues (Fig. 1B), in the endothelium, Zeb1 increase has been already detected when melanoma is still sensitive to treatment (2 weeks) (Fig. 2). This observation opened the question about the role of Zeb1 in this context. Since the PI agrees with reviewers that the focus just on Zeb1 might be risky, among the described activities, the PI planned, already in the original version of the present proposal, secretome and RNASeq analyses to identify novel melanoma vulnerabilities to be exploited in counteracting resistance to BRAFi and ICB therapy. In the present version of the proposal, PI clarified this aspect (see page 7). The PI strongly believes that this approach will reveal additional important factors contributing to endothelial-driven immunosuppressive profile and anti-cancer resistance in melanoma. Indeed, among milestones (M), M3 aims to identify molecular mediators of TEC plasticity and TEC-dependent immune evasion.

Q2 Reviewer #1: Ambitious project proposal and lack of in vivo analyses related to anergy and EndMT.

A2: Regarding concerns about the accomplishment of goals described in the research proposal, PI apologizes not to have sufficiently stressed that human and mouse samples described in WP1 Task1.1 are already in place and available to the PI (see page 7), which will perform immune-staining analyses. Similarly, inducible endothelial reporter mice are already available to the PI and will be exploited to analyze in vivo anergy and EndMT as planned in WP1 Task1.2 (see page 7-8). PI apologizes for the lack of clarity that prompted the reviewer to express a concern related to the absence of activities aimed to dissect in vivo anergy and EndMT. Indeed, Objectives of Task 1.2, entitled TEC in vivo analysis, are: a) to describe TEC features supporting melanoma stromal remodeling (Objective 2) and b) to characterize the TGFβ/Zeb1 circuitry involvement in TEC-driven resistance (Objective 3) providing an in vivo model to follow anergy and EndMT, which will possibly provide quantification of TEC-derived CAFs and hints on melanoma remodeling. For what concerns Zeb1 flox/flox mice, the PI will take advantage of a specific service at CNR-IBBC Monterotondo campus, which hosts an animal facility operating in accordance with the guidelines of the FELASA and routinely generating specific transgenic animals models by both standard (ESC blastocyst injection) and cutting-edge methodologies (i.e. CRISPR/Cas9 technology). The transgenic mouse facility is part of INFRAFRONTIER-Mouse Clinic European Research Infrastructure and at moment already produced about 140 mutant mouse lines and 50 mouse mutant CRE driver lines. The actual production capability of this team is about 15-20 mutant lines per years (http://www.ibcn.cnr.it/index.php/en/facilities/transgenic-facilities/monterotondo). For this reason the generation of Zeb1 flox/flox mouse will be conducted by experts working at the facility (see

collaboration letter signed by Dr Chiani and Dr Gambadoro) and will allow the PI´s team to conduct in the meanwhile the other activities described in the research plan. Contingency plan related to Zeb1 flox/flox mouse generation was envisaged (see pages 10-11). Research plan was modified according reviewer´s doubts on feasibility redesigning experiments. It now includes 2 WPs, each conveniently divided into 2 tasks.

Q3: Reviewer #1 and #2: Pertinence of in vitro model/overuse of HUVECs.

A3: The PI thanks the reviewers to point out doubts on the exploitation of HUVECs for in vitro experiments. The PI agrees with reviewers that RNASeq performed on these cells are risky. For this reason, in the revised version of the research proposal, RNASeq has been planned in tumor endothelial cells (TECs) isolated from melanoma tissues of responder and resistant endothelial reporter mice, whose isolation protocol has been established adapting protocol described in PMID:26554446. Original Task 2.1 related to studies on HUVECs has been removed and experiments of research plan re-designed. Anergy and EndMT molecular mechanisms will be studied in ECs isolated from melanoma tissue both of inducible endothelial reporter mice and of inducible endothelial Zeb1-KO mice. Specifically, isolated ECs from endothelial reporter mice will give information about TEC features supporting melanoma stromal remodeling (**Objective 2**). The comparison of these cells with the ones isolated from inducible endothelial Zeb1-KO mice will characterize the TGFβ/Zeb1 circuitry involvement in TEC-driven resistance (**Objective 3**). In the revised version of the present proposal WP1, Task 1.2 and WP2 have been modified according Reviewers´ suggestion (see pages 7-8).

Q4 Reviewer #1, #2, #3: PI cancer commitment.

A4: Relating to Reviewer concern about PI focus on the cancer field, the PI apologizes not to be sufficiently clear about it. She started her retraining in molecular oncology. This led to the publication of a co-last author paper about the role of Zeb1 in breast cancer metastasis on Cell Death and Disease in 2018 (PMID: 29988033) and to establish a collaboration with the Department of Oncology at the University of Turin, where she spent last year as visiting scientist. Moreover, she published a collaborative study analyzing the phenotype of fibroblasts in nodular sclerosing subtype of classical Hodgkin lymphoma on Cancers in 2019 (PMID: 31671543). At moment she submitted a review entitled “Perspective: is there a role of redox state in cancer metastatic cells?”, as first author, and a paper entitled “Metabolic re-programming by MALAT1 depletion in prostate cancer”, as contributing author. Based on these facts, the PI would strengthen her commitment to cancer research exploiting her past background on physio-pathology. The present proposal has been conceived exactly in this frame, the PI background in molecular mechanisms harnessing vasculature functions is the type of “hybrid vigor” one needs in scientific world and will have profound implications in the understanding of tumor endothelium role in the onset of acquired resistance in melanoma. In case of funding, MFAG will be of great value to consolidate PI research in molecular oncology.

Q5 Reviewer #2: PI no senior authorship

A5: PI would like to clarify that she published a co-last author paper about the role of Zeb1 in breast cancer metastasis on Cell Death and Disease in 2018 (PMID: 29988033).

Q6 Reviewer #2: Suggestion to get mentorship from collaborators working in the melanoma field.

A6: In the present version of the proposal, PI increased the number of collaborators opening the possibility to have mentors for each scientific aspect of the research plan. Indeed, 3 additional units have been included in the team: 1) a clinical expert in melanoma, Prof. Peris K., working at Policlinico Gemelli; 2) a scientist with a long-standing experience in tumor immunology and immunotherapy, Dr Nisticó (Regina Elena National Cancer Institute); 3) and Prof Bernardini actively working on leukocytes trafficking (Sapienza University) (see new collaboration letters). For collaborator expertise in cancer and melanoma field, please refer to the document entitled: “Description of work for each unit of personnel”, where relevant publications were listed.

Q7 Reviewer #3: CV only includes 16 publications.

A7: For what concerns doubts about PI´s publication record, PI would like to bring to the attention of Reviewer that the 16 publications listed in the proposal are related to last five years (from 2015 to 2020) of her scientific career as requested by AIRC into the MFAG call 2020. According Scopus, PI publication record counts 30 publications with an h-index of 17 since the beginning of her career in 2007.

1. IMPACT

The latest breakthroughs in the genetic and molecular biology of tumors opened the avenue to tailored therapies with impressive results. About 40% of human skin melanoma harbor **BRAF mutations** (1). Among them $BRAF^{V600E}$ has a prevalence over 90% (1). Recently, **BRAF mutation-targeted therapies** significantly improved progression-free survival of melanoma patients, but their efficacy is still limited because of widely variable responses and resistance onset (2). Indeed, 15% of BRAF mutant melanoma develops **primary resistance** to **BRAF inhibitors** (BRAFi), including vemurafenib, dabrafenib and encorafenib, and most responders experience **acquired resistance** (2). Mechanisms of **adaptive response to BRAFi** further limit their clinical efficacy so much that only 3-6% of melanoma patients shows complete response (3). The immunogenicity of melanoma and its response to immunomodulation generated great hope, however 40-65% of melanoma patients shows **primary resistance** to immune checkpoint blockade (ICB) therapy with Anti-PD1 (4). The percentage increases up to 70% when CTLA4 antibody is used as ICB approach. Among ICB responders, 43% develops **acquired resistance** in 3 years (4). The combination of targeted and immunotherapy is still under investigation: potential benefits cross concerns about toxicities and resistance (5). Thus, accurate understanding of the biological mechanisms underlying BRAFi and ICB resistance is an unmet need. **Tumor associated endothelial cells (TECs)** support tumor progression by *angiogenesis*, *anergy* and endothelial-to-mesenchymal transition (*EndMT*) (6,7). How these processes influence anti-neoplastic therapy response has been only partially characterized. Based on the crucial role of melanoma vasculature in the stromal remodeling and immune cell infiltration (6,7), **we hypothesize that TECs represent common mediators for the onset of resistance to BRAFi and ICB therapies.** Transforming growth factor β (**TGFβ**), which is increased by BRAFi treatment, deeply regulates TEC functions (8,9). Indeed, TGFβ promotes tumor growth by alteration of vascular functions, inhibition of immune surveillance and establishment of a tumor microenvironment (TME) supporting cancer growth and therapy evasion (9). In this light, here **the overarching goal is to elucidate TGFβ-driven TEC alterations in melanoma stromal remodeling and immune evasion and how they contribute to resistance to BRAFi and ICB therapies alone or in combination.** The present proposal aims to fulfill the knowledge gap on an overlooked biological process, the role of TECs, **promising the development of new combinatorial therapies with increased clinical efficacy able to prevent/delay melanoma resistance.**

2. RATIONAL AND FEASIBILITY

2.1 STATE OF THE ART

2.1.1 TGFβ and melanoma. High levels of TGFβ in the TME and the activation of its downstream pathways associate with poor prognosis in cancer (8,9). TGFβ mediates tumor progression by paracrine and autocrine action, favoring angiogenesis, stromal remodeling, immunosuppression and invasion (9,10). Its effects on stromal activation and immunosuppression detrimentally influence melanoma response to BRAFi and immunotherapy and contribute to resistance onset (9). Intriguingly, upon BRAFi treatment melanoma cells release high levels of TGFβ driving a TME remodeling responsible for a stroma supporting therapy evasion (10,11). Moreover, constitutive activation of TGFβ signaling in TME causes tumor leukocyte exclusion and consequent poor response to ICB therapy (9). These evidences support the hypothesis that TGFβ exerts a crucial role in resistance to anti-neoplastic therapies. Nevertheless, the specific effect of a TGFβ rich TME on TECs has not been analyzed yet. **The involvement in the modulation of tumor stroma and in the immune cell recruitment puts TECs at the crossroad for the interpretation of molecular mechanisms involved in therapy resistance, opening the avenues to new combinatorial treatments aimed to increase therapy efficacy in melanoma patients.**

2.1.2 TGFβ/Zeb1 circuitry mediates EndMT. TGFβ induces mesenchymal transition of TECs activating a series of signaling pathways converging in the transcriptional regulation of mesenchymal markers and growth factors (12). TGFβ directly controls the activity of the EndMT master regulator Zeb1, which orchestrates the progressive loss of endothelial features, including cell-cell contact and expression of endothelial-specific markers (like VE-cadherin), and the parallel acquisition of mesenchymal phenotype (12). Specific EndMT-inducing transcription factors, Zeb1 and Twist, play a pivotal role in melanoma progression, promoting cell transformation, carcinoma metastatic dissemination and resistance to treatment (13,14). Interestingly, in BRAF mutant melanoma, high Zeb1 expression levels is typical of aggressive melanoma, correlate with poor prognosis and associates with a phenotype switching conferring chemoresistance by drug adaptation and an

immunosuppressive profile (14-17). Indeed, Zeb1 transcriptionally controls IL-2 expression, the key cytokine of T cell functions (18). Nevertheless, at present no investigation described Zeb1 role in endothelium-driven resistance to anti-cancer therapy. Recent reports showed that about ~40% of cancer associated fibroblasts (CAFs) arises from TECs undergoing EndMT (6). Synthetically active CAFs support tumor growth, metastasis dissemination and extracellular matrix deposition contributing to TME remodeling, immune escape and therapy resistance (19,20). In BRAF mutant melanoma, BRAFi treatment activates fibroblastic stroma to generate a fibronectin-rich extracellular matrix, which provides signals for BRAFi tolerance (19). Hence, **the analysis of TGFβ/Zeb1 circuitry in TEC plasticity promises to shed light on one of the molecular mechanisms contributing to the onset of melanoma resistance to be exploited in an anti-cancer perspective.**

2.1.3 EC anergy limits anti-tumor response. Primary resistant melanoma to immunotherapy show low levels of infiltrating immune cells (7). Immune cell recruitment is regulated by the expression of adhesion molecules and chemokines on the vasculature, a process known as "endothelial activation" crucial for capture, rolling and transmigration of leukocytes from the blood to the site of tissue injury (7). Leukocyte recruitment upon endothelial activation and subsequent trans-endothelial migration depends on the interaction between proteins expressed on leukocytes and active endothelial cells (7). TME constitutively secretes pro-angiogenic factors, which detrimentally affect vasculature morphology and function (7). The angiogenic switch leads to an inefficient response of TECs to pro-inflammatory factors and a consequent insufficient expression of molecules involved in capture, adhesion and extravasation (7). The missing endothelial activation leads to EC anergy that effectively dampens anti-tumor response (7). Beyond the regulation of immune cell recruitment, TECs actively contribute to counteract anti-tumor response also by expression of inhibitory molecules including ICB (PD-L1, B7-H3 and B7-H4), death receptor-ligands (TRAIL, FasL) and secreted immunomodulatory factors (IL-6, PGE, IL- 10 and TGFβ) (7,21). In this light the contribution of TEC in the modulation of anti-tumor response and in the response to immunotherapy needs appropriate investigation as **specific therapeutic approaches aimed to alleviate EC anergy could enhance leukocyte recruitment in tumors and favor immunotherapy response.**

2.2 QUESTIONS

The present proposal aims to provide an answer to the following key questions: 1) *Do TECs contribute to melanoma resistance to BRAFi and ICB therapy?* 2) *Is the TGFβ/Zeb1 circuitry involved in TEC-driven stromal remodeling and immune escape?* 3) *Are small molecules interfering with TGFβ/Zeb1 circuitry able to prevent/delay melanoma resistance to BRAFi and ICB therapy?*

2.3 DRIVING HYPOTHESIS

The driving hypothesis of the present proposal is that melanoma associated endothelial cells undergo EC anergy and EndMT contributing to the onset of melanoma stromal remodeling, immune evasion and thus resistance to BRAFi and ICB therapy. Indeed, the abundance of proliferative stimuli and TGFβ in melanoma could prevent endothelial activation reducing immune cell recruitment *(EC anergy)* and induce *EndMT* favoring the development of CAF-dependent melanoma resistance. High TGFβ levels associate with melanoma relapse (9,11) and elevated Zeb1 levels correlate with drug adaptation (14-17). We hypothesize that TGFβ/Zeb1 circuitry could be one of the mechanisms harnessing both TEC anergy and EndMT and thus playing a role in melanoma progression and resistance. Consequently, we speculate that inducible endothelial Zeb1 knockout mice will highlight Zeb1 role in TECs and that small molecules interfering with TGFβ/Zeb1 circuitry will delay/prevent melanoma resistance to BRAFi and ICB therapy alone or in combination.

2.4 OBJECTIVES

The **primary objective** is characterizing the role of TEC in the onset of melanoma resistance to shed light on the main molecular determinants exploitable in novel combinatorial anti-neoplastic approaches. **Key secondary objectives are:** 1) Immunophenotype characterization of responder and resistant melanoma; 2) Description of TEC features supporting stromal remodeling, immune evasion and melanoma progression; 3) Characterization of TGFβ/Zeb1 circuitry involvement in TEC-driven melanoma resistance; 4) Characterization of Zeb1 contribution to TEC function by an inducible endothelial Zeb1 knockout mouse; 5) Identification of small molecules interfering with TGFβ/Zeb1 circuitry able to favor melanoma response to BRAFi and ICB therapy alone or in combination.

2.5 PRELIMINARY DATA

2.5.1 Zeb1 INVOLVEMENT IN MELANOMA RESISTANCE TO BRAFi. The therapeutic effect of PLX4720, an analog of vemurafenib, was evaluated in mice injected with D4M cells, a BRAF-mutated melanoma cell line, over 6 weeks. During the first 2 weeks of treatment, PLX4720 treated mice show an initial delay of melanoma outgrowth in comparison to vehicle group. The early treatment time point suggests a response to therapy. Thereafter, melanoma gradually reached a tumor volume comparable to vehicle group within 6 weeks suggesting the development of acquired resistance to the drug (Fig. 1A). Notably, Zeb1 expression level significantly increases throughout the whole melanoma tissue of PLX4720-treated mice at 6 weeks compared to vehicle group (6-weeks) and PLX4720-treated mice at 2 weeks (Fig. 1B).

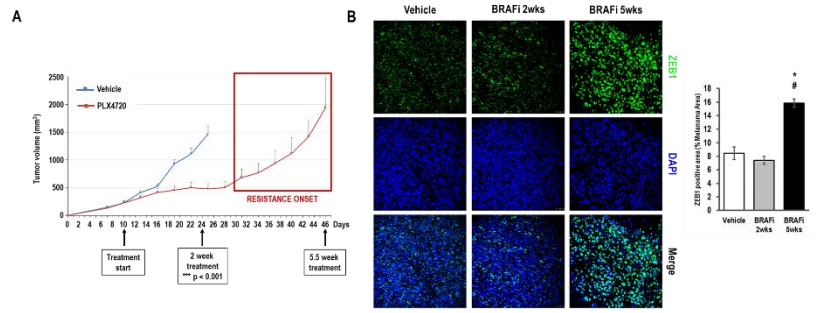


Fig. 1 PLX4720 therapeutic effect. A) *Tumor growth of mice bearing D4M cells were treated with vehicle (n=7; blue line) or PLX4720 (n=7; red line).* ***B)*** *Left panel: Representative confocal images of melanoma tissues treated for 2 or 6 weeks with PLX4720 probed with anti-Zeb1 (green). Nuclei counterstained with DAPI. Vehicle used as solvent control. Magnification 40X. Right panel: Quantification of Zeb1 positive nuclei.*

Specific analysis of melanoma endothelium ($CD31^+$ cells) pointed out a significant increase of $Zeb1^+$ nuclei upon PLX4720 treatment both at 2 and 6 weeks (Fig. 2). Data depicted in Fig. 1 and 2 point out Zeb1 sensitivity to BRAFi treatment indicating that its expression increases already during response to the drug (2 weeks) in case its expression into the endothelial compartment is specifically monitored.

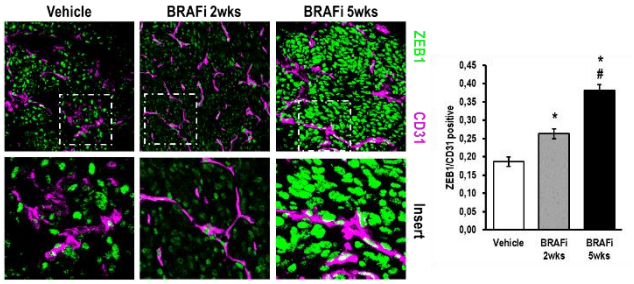


Fig. 2 PLX4720 effect on melanoma endothelium. *Left upper panels: Representative confocal images of melanoma tissues treated for 2 or 6 weeks with PLX4720 probed with anti-CD31 (magenta) and anti-Zeb1 (green). Vehicle used as solvent control. Magnification 40X. Left lower panels: Insets contain enlargements of the selected areas (dashed squares). Right panel: Quantification of Zeb1/CD31 positive nuclei.*

These preliminary analyses provide a rational for the investigation of the Zeb1 role in the onset of acquired resistance. At moment we analyzed PLX4720 treated mice, but we will extend our analyses to mice treated with Anti-PD1 or PLX4720+Anti-PD1 combination in WP1 TASK 1.1 – Subtask 1.1.2 to examine in greater details Zeb1 role in TECs during resistance onset.

2.5.2 Zeb1 EXPRESSION IN MELANOMA CELLS UPON BRAFi TREATMENT. Our preliminary data suggest Zeb1 involvement in BRAFi resistance (Fig 1 and 2) in agreement with recent published data describing Zeb1 role in melanoma cell plasticity and consequent resistance to MAPKi (14). These evidences put Zeb1 under the spotlight as a player of tumor resistance. The analysis of mechanisms underpinning Zeb1 mediated resistance promises **the identification of strategies aimed at maintaining Zeb1 expression at low levels during anti-neoplastic treatments**. This perspective is supported by the observation that highly expressing Zeb1 melanoma cells respond to BRAFi treatment down-regulating Zeb1 (Fig. 3), indicating that BRAFi regulates Zeb1 activity. Moreover, one of the members of melanocyte inducing transcription factor (MiTF) family, TFEB, whose transcription is affected during BRAF-driven melanoma progression and chemo-resistance (22), showed sensitivity to BRAFi treatment.

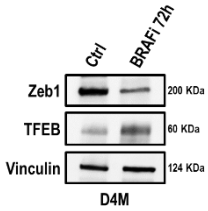


Fig. 3 BRAFi decreases Zeb1 expression and enhances TFEB levels. *D4M cells were treated with BRAFi 0.5μM for 72 hours (h), lysed in LAEMMLI buffer and western blot for Zeb1 and TFEB was performed. Solvent-treated cells used as reference. Vinculin used as protein loading control.*

These data provide a rational to investigate whether BRAFi resistance in melanoma associates with a bypass of BRAFi-driven Zeb1 inhibition and consequent Zeb1-dependent tumor progression (activities planned in WP2 TASK 2.2 – Subtask 2.2.1).

2.5.3 AA6, MS-275 AND LY2157299 TREATMENT ALONE OR IN COMBINATION WITH BRAFi. TGFβ and Zeb1 play a detrimental effect on anti-neoplastic therapy favoring drug resistance (9,10,14). To counteract their activity, we tested in melanoma cells the effect of small molecules interfering with TGFβ/Zeb1 circuitry. The lack of a specific Zeb1 inhibitor prompted us to test small molecules indirectly hindering Zeb1 activity, including AA6, a DNA methylation inhibitor, and MS-275, a specific inhibitor of class I HDACs (23-25).

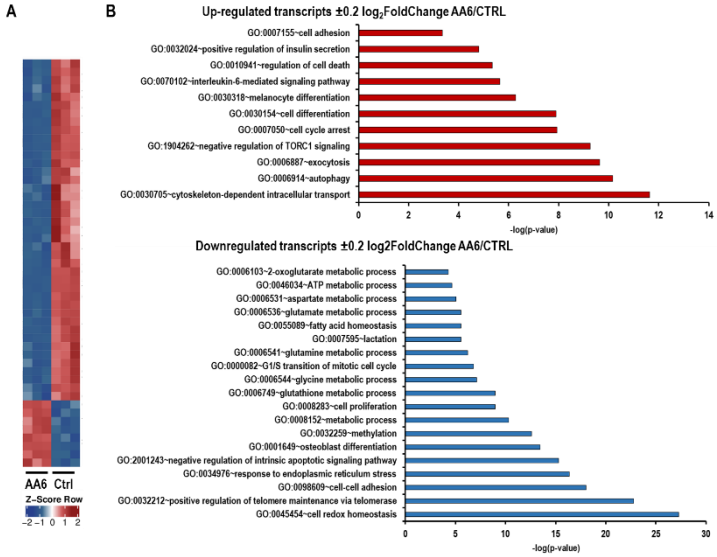


Fig. 4 AA6 anti-neoplastic properties. **A)** *Heatmap showing the 50 most differentially regulated genes in B16 treated 48h with AA6 50μM identified by total RNA-Seq analysis (n = 3 each group). Red and blue represent over- and under-expressed genes, respectively.* **B)** *Gene ontology analysis of AA6-differentially regulated transcripts. Upregulated genes depicted in the upper panel, red bar graph; downregulated genes in the lower panel, blue bar graph.*

The PI demonstrated the anti-neoplastic potential of the small molecule AA6 and its ability to down-modulate Zeb1 in a mouse model of breast cancer-associated lung metastasis (25). Here, melanoma B16 cells, treated for 48h with AA6 50μM, were analyzed by RNASeq. The gene ontology (GO) analysis of differentially expressed transcripts (Fig. 4A), performed by DAVID, pointed out AA6 anti-neoplastic properties also in mouse melanoma cells (Fig. 4B). Specifically, AA6 treatment induces the expression of transcripts involved in melanocyte differentiation, cell differentiation and cell cycle arrest and inhibits the expression of transcripts involved in cell proliferation, metabolic processes and in the positive regulation of telomere maintenance (Fig. 4B). Interestingly, invasiveness assay performed on B16 cells showed an impairment of cell migration into matrigel in response to AA6 (Fig. 5).

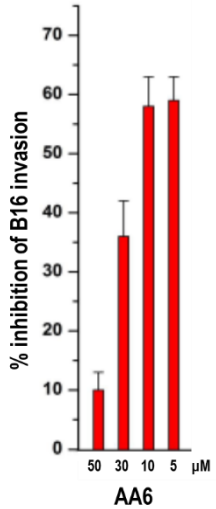


Fig. 5 Effect of AA6 on B16 invasion properties. *Percentage of invasion inhibition o fB16M cells treated for 20h with AA6 50, 30, 10, or 5 μM. Solvent-treated cells used as reference. Data are presented as mean ± SE.*

This preliminary analysis sheds light on AA6 as a drug with interesting clinical potential. Moreover, in association with BRAFi, AA6 strongly down-modulated Zeb1 expression in D4M cells (Fig. 6), providing a rational to investigate whether it can enhance melanoma response to BRAFi and ICB therapy as planned in WP2 TASK 2.2 – Subtask 2.2.2. MS-275 has been chosen because class I HDACs: 1) bind Zeb1 in transcriptional repressor complexes (24) and 2) related inhibitors improve Anti-PD1 response (23). MS-275 is able to completely change the morphology of melanoma cells towards a stellate phenotype without affecting cell viability (data not shown; cell viability after 72h of treatments 92±2%) and decreases Zeb1 expression. Moreover, the inhibitor of the TGFβ receptor type 1 kinase, LY2157299, was tested for its known anti-cancer activity (26). Interestingly, its combinations with BRAFi showed promising inhibitory activity on Zeb1 expression, paralleled by increased TFEB expression (Fig. 6). Altogether, these evidences support experiments planned in WP2 TASK 2.2 – Subtask 2.2.1.

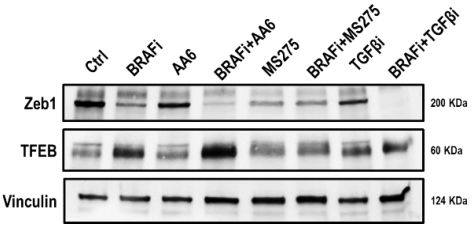


Fig. 6 Effect of TGFβ/Zeb1 circuitry interfering in the presence/absence of BRAFi. *D4M cells were treated for 72h with BRAFi 0.5μM, AA6 50μM, MS275 20μM or LY2157299 20μM lysed in LAEMMLI buffer and western blot for Zeb1 and TFEB was performed. Solvent-treated cells used as reference. Vinculin used as protein loading control.*

2.6 METHODOLOGIES

2.6.1 Patient samples. A cohort of 30-40 melanoma patients diagnosed and followed-up at the Candiolo Cancer Institute and Policlinico Gemelli will be included in the study as a reference human tissue bank. The study

protocol is in the process to be approved by the internal ethic committee. Patients were classified on the basis of AJCC criteria and treated and followed-up according standard guidelines (27,28). The study cohort consisted of melanoma bearing $BRAF^{V600E}$ mutation treated with BRAFi, Anti-PD1 or both in combination. Specimens were formalin-fixed, paraffin embedded, and 3 µm-thick tissue sections were cut. Sections underwent for routine haematoxylin and eosin staining. Then, histopathologic analysis was performed by experienced dermatopathologists. Characteristics of the patients, the primary tumors and follow-up data were collected. The recorded parameters are: age, sex, site of primary melanoma, histological type, Breslow thickness, Clark level and response to therapy.

2.6.2 Cell lines. *In vitro* experiments will be performed comparing cell functions in all the melanoma cell lines listed in table below. *In vivo* experiments will be conducted using Mel-5555 and D4M cells, two melanoma cell lines bearing orthologous human BRAF mutation, sensitive to PLX4720 and well recapitulating human disease.

Species	Name	Mutation pattern
Mouse	B16	BRAF wt/wt
Mouse	Mel-5555	BRAF mut/wt
Mouse	D4M	BRAF mut/wt; PTEN -/-
Human	SK-MEL5	BRAF mut/wt
Human	A375	BRAF mut/mut; NRAS mut/wt; MAP2K1 mut/wt

Experiments on ECs will be performed on ECs isolated from mouse melanoma samples from responder and resistant mice by immune-magnetic enrichment of $CD31^{+}$ cells (29). Leukocytes derived from peripheral blood mononucleated cells (PBMCs) will be used in trans-endothelial migration assays for EC anergy evaluation in collaboration with Dr Bernardini.

2.6.3 Mouse models. iEC^{tomato} mice. Inducible EC reporter mice, in which endothelial cells are irreversible tagged by dTomato, will be obtained after mating endothelial inducible mice (Cdh5-CreERT2 mice) with reporter mice (R26Rosa-lox-Stop-lox-tdTomato-hrLuc mice) both already available in the lab. $Zeb1^{iEC-/-}$ (B6 - $Zeb1^{<tm1a(MFAG)Cnrm>}$ Tg(Cdh5-cre/ERT2)1Rha/Cnrm) mice. Inducible endothelial Zeb1 knock-out (KO) mice will be generated by Dr Chiani and Dr Gambadoro working at the Transgenic mouse facility of CNR (see collaboration letter). They will exploit the technique of targeted homologous recombination in murine embryonic stem cells (mESCs) through electroporation of an efficient Zeb1 targeting construct. Since Zeb1 knockout mice are perinatal lethal (30,31), a Cre/loxP technology will be adopted to generate conditional Zeb1 knockout mice. Specifically, two loxP sites, 34 bp nucleotide sequences, will be inserted in the construct around an essential exon of Zeb1, according standard procedures (32,33). Then, electroporated mESCs will be microinjected into mouse blastocysts (34). The obtained engineered blastocysts will be transferred into the uterus of pseudopregnant recipients, which will give birth to chimeric mice. Thereafter, mice harboring floxed Zeb1 allele in all tissues, but phenotypically WT, will be derived from chimeric mice. $Zeb1^{flox/flox}$ mice will be then bred to Cdh5-CreERT2 mice (already available in the lab), wherein the endothelial specific promoter Cdh5 will determine the site of the gene deletion into the endothelium. Melanoma mouse model. BRAF mutant melanoma mouse model will be realized in the following 6-week-old immune-competent C57BL/6 mouse strains: 1) iEC^{tomato}; and 2) $Zeb1^{iEC-/-}$. These mice will be subcutaneously (sc) injected with a syngeneic Mel-5555 ($5x10^5$ cells) or D4M ($5x10^5$ cells) cell suspension of PBS and matrigel (1:1). Tumor outgrowth will be monitored bi-weekly until a palpable mass will be identified, then tumor size will be measured daily with a caliper. Tumor volume will be calculated by the modified ellipsoid formula: (length x $(width)^2$)/2. When tumors will reach a volume of approximately 250–300 mm^3, mice will be randomly assigned to different treatment groups and maintained for 2 weeks (short-term trail) or until tumor will reach 2000 mm^3 as a consequence of therapy resistance (long-term trial). During randomization, mice with a tumor size lower or higher than 250–300 mm^3 will be excluded. Groups will be treated as follow: 1) controls (equivalent volume of vehicle); 2) Anti-PD1 (12,5 mg/Kg intraperitoneal (ip) injection every 3 days diluted with InVivoPure pH 7.0 Dilution Buffer (BioXCell)); 3) PLX4720 (BRAFi) (daily oral gavage at 60 mg/kg, dissolved in a vehicle of 1% w/v methylcellulose in sterile water); 4) PLX4720 + Anti-PD1 (COMBO); 5) the most promising TGFβ/Zeb1 circuitry inhibitor according results derived from WP2 TASK 2.2 – Subtask 2.2.1; and 6) COMBO + TGFβ/Zeb1 circuitry inhibitor. Mice not experiencing resistance and showing regression of tumor size (responder to therapy) will be euthanized after 10 weeks of treatment for subsequent analyses. Melanoma samples will be collected fresh and/or harvested both in OCT and in vital freezing medium.

2.6.4 Evaluation of tumor immune cell infiltration. Immune cell infiltration will be evaluated by immune-staining of human and mouse melanoma sample slices and of fresh or vital frozen samples from mouse melanoma. Melanoma sample slices will be evaluated by immunofluorescence confocal analysis (Leica SPE confocal laser-scanning microscope), whereas fresh or vital frozen samples will be analyzed by

immunofluorescence cytofluorimetry analysis (Beckman Coulter CyAn™ ADP Analyzer). The following markers will be considered to evaluate the composition of immune infiltrate: T cells ($CD45^+CD3^+$; $CD45^+CD3^+CD4^+$; $CD45^+CD3^+CD8^+$; $gdTCR^+$), B cells ($CD45^+CD19^+$) myeloid derived suppressors cells ($CD11b^+Ly6G^{high}Ly6C^{low}$; $CD11b^+Ly6C^{high}Ly6G^{low}$), macrophages ($CD11b^+F4/80^+Ly6C^+$), neutrophils ($CD11b^+Ly6G^+$), dendritic cells ($CD11c^+MHCII^+$), M1-macrophage ($CD11b^+Ly6C^+iNOS^+$; $CD11b^+Ly6C^+$), M2-macrophage ($CD11b^+Ly6C^+CD206^+$), NK cells (NK1.1; NKp46; CD11b; CD49b; CD27); Treg ($CD4^+$, $Foxp3^+$). Immune exhaustion/activation will be analyzed assessing the expression of: PD-1, CTLA4; LAG3, 2B4, TIM3, BTLA, TIGIT, Sca1, CD69, CD25 4-1BB, TCF1, KLRG1.

2.6.5 Analysis of EC anergy. TEC anergy will be evaluated *ex vivo* in ECs isolated from mouse melanoma samples of responder and resistant mice by immune-magnetic enrichment of $CD31^+$ cells in the presence or absence of TGFβ and/or TNFα. Flow cytometry analysis will reveal the expression levels of molecules involved in leukocyte recruitment. Specifically, the following markers of capture, rolling and arrest phase in leukocyte recruitment will be evaluated: P-selectin, E-selectin, ICAM1, VCAM1 and ICAM2, VE-cadherin, JAMs, ESAM, CD31, CD99, CD99L2 and JAMA. The involvement of TGFβ/Zeb1 circuitry in EC anergy will be evaluated by transendothelial migration assays (35). Specifically, a monolayer of isolated mouse TECs will be prepared 48h before the assay by plating $2x10^4$ cells in 24-well Boyden's chambers. Then, migration of $5x10^5$ PBMCs towards mouse BRAF mutated melanoma cells will be evaluated after 5h. The number of migrating cells will be determined. The migration index (MI) will be calculated by dividing the number of cells which migrated in the presence of melanoma cells by the number of cells which migrated in response to medium alone. Similar analysis will be conducted in the presence of small molecules interfering with TGFβ/Zeb1 circuitry in combination with BRAFi, including TGFβ inhibitor (LY2157299); or Zeb1 indirect inhibitors (AA6; and MS275).

2.6.6 Analysis of EndMT. The determination of the percentage of CAFs derived from TECs will be evaluated exploiting iEC^{tomato} mice, where ECs are irreversibly tagged by dTomato. This mouse model will allow revealing the percentage of CAFs double positive for Fibroblast activation protein (FAP) and dTomato deriving from EndMT displaying TEC contribution to stromal compartment. Specific EndMT markers will be evaluated ex vivo by immune-staining (WB or immunofluorescence). Among them, early EndMT markers: downregulation of CD31, VE-cadherin and dismantled adherens junctions; upregulation of αSMA, SM22α, FSP1, CD44; and late EndMT markers: upregulation of SM-SMHL, SM-calponin, smoothelin, fibronectin, tenascin, collagen III, THY1, Vimentin, Notch3, PAI, Sca1, MMP2 and 9, VCAM and ICAM1 will be evaluated. Moreover, the expression of specific EndMT transcription factors will be assessed, including Zeb1, Twist, Snail, Slug and fibrotic markers. The expression of these genes will be also evaluated by qRT-PCR. Similar analysis will be conducted in the presence of the aforementioned small molecules interfering with TGFβ/Zeb1 circuitry in combination with BRAFi.

2.6.7 Secretome analysis. Plasma and serum derived from control and treated iEC^{tomato} mice and supernatant of isolated melanoma ECs will undergo secretome analysis by a Luminex xMAP multiplex bead-based assay approach, using antibody panels for Cancer Biomarkers (R&D Systems 10-plex: Fas Ligand; basic FGF; HGF; IL-6; CXCL10; Leptin; Osteopontin; Prolactin; TNFα; VEGF) and for Immuno-Oncology Checkpoint (R&D Systems 20-plex: GM-CSF; Granzyme B; IFNγ; IL-1α; IL-1β; IL-10; IL-12 p70; IL-13; IL-17A; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; MCP-2/CCL8; MCP-3/CCL7; MICA; MIP-1α/CCL3; MIP-1β/CCL4).

2.6.8 TEC-associated signature of melanoma resistance. RNASeq. Total RNA from ECs isolated from mouse melanoma samples derived from responder and resistant mice will be prepared by Maxwell RNA extraction kit and subjected to high-throughput sequencing. Three biological replicates will be analyzed. The integrity of RNA will be checked on Bioanalyzer, and 5μg of RNA with RIN>9 will be used for ribosomal depletion using the Ribo-Minus Eukaryote Kit v2. Thereafter, a standard RNA-seq Library protocol will be performed. Obtained RNA libraries will be quantified on Qubit 2.0 and diluted for template preparation. RNA sequencing will be performed in an Illumina NextSeq500 sequencer, obtaining a mean of 75 million 75 bps-single reads per sample, with a standard protocol. The Bowtie program (36) will be used to align reads to the reference mouse mm10 genome. Annotations provided by Ensembl GRCm38 and GENECODE M25 will be set as reference for the RSEM computational pipeline (37) used for quantification of gene expression levels. The EBseqtool (38) will be used to evaluate modulated genes with FC>2 and FDR<0.1 as parameters to define the statistical significance of differential gene expression. NGS-Seq data will be deposited to the GEO database (https://www.ncbi.nlm.nih.gov/geo/) upon publication.

2.7 POWER AND STATISTICAL ANALYSIS

Patient samples. Characteristics of the patient (age and sex), primary melanoma (site, histological type, Breslow thickness, Clark level, ulceration, mitotic rate and regression), entity of the immune infiltration, presence of EndMT and TEC anergy will be analyzed in 30-40 patients by a Chi-squared test for univariate analyses and logistic regression analysis for the multivariable model identifying significant associations with a p-value <0.05 and a power=0.8. Mouse experiments. Considering a number of 10 mice for each treatment, a standard deviation of σ=15% (due to ± 15% of spontaneous different tumor sizes), a statistical significance of p = 0.05 (a significant event is an event with a 5% probability to occur by chance), then the probability to identify all true positive events is 85% (that is to say that experimental power will be 0.85). Since usually effect below 20% could be considered barely relevant, our work plan will have a potential to identify all significant events with a power of 85%.
The Gpower software will be used to determine the correct sample size for our experiments (http://www.gpower.hhu.de/en.html). Statistical analyses will be conducted using GraphPad Prism 6 software adopting the correct statistical test according to the characteristics of the experiment analyzed (parametric or non-parametric tests). Data will be presented as mean±sem and a p<0.05 will be considered significant. NGS sequencing data will be analyzed using appropriate bioinformatics tools for statistical purposes.

3. RESEARCH PLAN

The present project will be organized in 2 interconnected work packages (WPs).
WP1. Assessment of TEC contribution to melanoma resistance. The role of TECs in melanoma therapy resistance will be assessed *ex vivo* in human and mouse melanoma samples derived from responders and non-responders to BRAFi and ICB therapy and *in vivo* in an endothelial reporter mouse model bearing BRAF mutant melanoma upon treatment with BRAFi, Anti-PD1 or both in combination. Ex vivo analysis in isolated ECs will be exploited to investigate the role of TGFβ/Zeb1 circuitry in TEC anergy and EndMT.
WP2. Targeting of TGFβ/Zeb1 circuitry to overcome TEC-driven melanoma resistance. Genetic and pharmacological targeting of TGFβ/Zeb1 circuitry will be tested to verify whether this strategies might delay/prevent the onset of melanoma resistance to BRAFi and ICB therapy.

3.1 WP DESCRIPTION

WP 1. ASSESSMENT OF TEC CONTRIBUTION TO MELANOMA RESISTANCE (Mo: 1-36)**.**
Objectives- TECs are really active component of TME and strongly contribute to tumor progression (6,7,11). We speculate that TEC are involved in the mechanisms mediating stromal remodeling and immune escape responsible of melanoma resistance. To clarify the correlation among the activation of TECs, EndMT, immune response and melanoma survival/stage/recurrence, studies will be performed *ex vivo*, both in a cohort of melanoma patients, stratified by disease stage and follow-up data, and in murine melanoma samples derived from responder and resistant mice treated with PLX4720, Anti-PD1 and COMBO. The results obtained will be paralleled and matched taking advantage of an endothelial reporter mouse model injected with syngeneic BRAF mutant melanoma during response and progression to PLX4720, Anti-PD1 and COMBO therapy. The analyses will focus on the presence of mediators of immune response and on the expression level of markers involved in adhesion, rolling, transendothelial migration of leukocytes and in EndMT.
Task 1.1: TEC ex vivo analysis (Mo: 1-12). Task 1.1 will be conveniently brought down into sub-tasks. Task 1.1 will provide: a) an immune-phenotype characterization of melanoma responders and non-responders to BRAFi and ICB therapy (**Objective 1**); and b) insight into the status of TEC in melanoma (**Objective 2**).
Subtask 1.1.1: Human samples. Dr Sapino (Candiolo Cancer Institute) and Dr Peris (Policlinico Gemelli) will provide melanoma biopsies already in place derived from a cohort of 30-40 melanoma patients (stratified by disease stage and follow-up data). Melanoma sample slices will be analyzed for the entity of tumor-infiltrating leukocytes and markers of immune activation/exhaustion, endothelial activation and EndMT. The acquired data will be then matched with clinical and histopathological information.
Subtask 1.1.2: Mouse samples. Similar investigation will be conducted on already available melanoma samples derived from mice bearing orthologous human BRAF mutated melanoma by sc injection of syngeneic Mel-5555 or D4M melanoma cells provided by Dr Bussolino. Melanoma samples have been harvested after treatment with PLX4720, Anti-PD1 and COMBO at 2 weeks (therapy response) and 6 weeks, when resistance occurs, and stored for immunofluorescence analyses by confocal and flow cytometry. Melanoma samples will

be evaluated for the entity of tumor-infiltrating leukocytes and for markers of endothelial activation, EndMT, immune activation/exhaustion. Data derived from the analysis will be matched with the ones obtained in Subtask 1.1.1 to highlight immunophenotype and TEC phenotype associated with melanoma resistance.

Task 1.2: TEC in vivo analysis (Mo: 7-36). The endothelial reporter iEC^{tomato} mice bearing BRAF mutated melanoma will be treated with PLX4720, Anti-PD1 and COMBO. Fresh melanoma samples will be collected at 2 weeks (response) and 6 weeks (progression) and analyzed directly or following harvesting in OCT or vital freezing medium. Specifically, exploiting iEC^{tomato} mice, in which TECs will be irreversibly tagged by dTomato, we will determine the percentage of CAFs deriving from TECs by confocal analysis of dTomato, FAP, a specific CAF marker, and EndMT markers. The effect of treatments on this percentage will be evaluated during response and progression. Blood and plasma derived from responder and resistant mice will undergo TGFβ level quantification (ELISA) and secretome analysis to gain insight into mediators of TEC plasticity and TEC-dependent immune evasion. We expect to find high levels of TGFβ, a molecule harnessing EndMT and increasing during melanoma resistance (10,11,26) and to highlight crucial secreting molecules involved in TEC anergy and EndMT. Moreover, ECs will be isolated from this mouse melanoma model by immune-magnetic enrichment. Thereafter, molecular mechanisms of TEC anergy and EndMT will be dissected to point out the TGFβ/Zeb1 circuitry connection with TEC anergy and EndMT (**Objective 3**). TECs isolated from responder and resistant mice will be analyzed for levels of released TGFβ, EC anergy and EndMT (see methodology section). Furthermore, an RNASeq analysis will be conducted in TECs isolated from responder and resistant mice to reveal mediators of TEC-driven acquired resistance. Subsequent bioinformatics integrative analysis of secretome and RNASeq analyses will allow to identify a TEC-dependent signature of resistance providing novel endothelial mediators of melanoma resistance to BRAFi and ICB therapy to be exploited as readout for our activities aimed to revert TEC phenotype responsible of melanoma resistance (see Task 2.1 and 2.2). Moreover, this approach will possibly identify novel melanoma vulnerabilities to be exploited in counteracting resistance to BRAFi and ICB therapy. Task 1.2 will further describe TEC features supporting melanoma stromal remodeling (**Objective 2**) and will characterize the TGFβ/Zeb1 circuitry involvement in TEC-driven resistance (**Objective 3**) providing: a) an *in vivo* model to follow EndMT and quantify the percentage of TEC-derived CAFs and b) a list of melanoma secreted molecules and transcripts driving TEC phenotype responsible of melanoma resistance to BRAFi and ICB therapy (TEC-dependent signature of melanoma resistance).

WP 2. TARGETING OF TGFβ/Zeb1 CIRCUITRY TO OVERCOME TEC-DRIVEN MELANOMA RESISTANCE (Mo 13-60)**.**

Objectives- TGFβ/Zeb1 circuitry supports cell dedifferentiation and metastasis dissemination (12-14). TGFβ levels increase during melanoma relapse (10,11,26). TGFβ drives Zeb1 expression, the master transcription factor of EndMT (12). We speculate that targeting of TGFβ/Zeb1 circuitry will reduce the incidence of BRAFi and ICB therapy resistance. The potential of this approach will be evaluated taking advantage of results obtained in WP1. The effect of Zeb1 blockade will be evaluated in an inducible endothelial Zeb1 KO mouse and translated to a preclinical model using small molecules interfering with TGFβ/Zeb1 circuitry in combination with COMBO. We expect that Zeb1 blockade in the endothelium will enhance melanoma BRAFi and ICB response preventing/delaying resistance onset with a high impact on melanoma patients. Our results will provide novel targets to revert TEC phenotype and will point out the relevance of TGFβ/Zeb1 circuitry in melanoma resistance.

Task 2.1: Analysis of Zeb1 knockout (KO) in TECs (Mo: 12-54). Zeb1$^{iEC-/-}$ mice, generated from Dr Chiani and Dr Gambadoro, will be sc injected with syngeneic BRAF mutant melanoma cells and treated with PLX4720, Anti-PD-1 and COMBO. Tumor outgrowth and response to therapy will be monitored. Melanoma samples will be collected after 2 and 6 weeks and analyzed for tumor-infiltrating leukocytes and markers of immune activation/exhaustion, endothelial activation and EndMT. Moreover, ECs will be isolated from mouse melanoma samples and analyzed for TEC-dependent signature (Task 1.2) by qRT-PCR. Then, TGFβ levels and the levels of secreting molecules highlighted in Task 1.2 will be measured in blood and plasma of Zeb1$^{iEC-/-}$ mice and in the supernatants of isolated melanoma EC. Data obtained from Zeb1$^{iEC-/-}$ mice will be compared with the ones obtained in Task 1.2 to dissect Zeb1 contributive role to TEC-driven melanoma resistance to BRAFi and ICB therapy revealing a Zeb1-dependent signature of TEC-driven acquired resistance in melanoma (**Objective 4**).

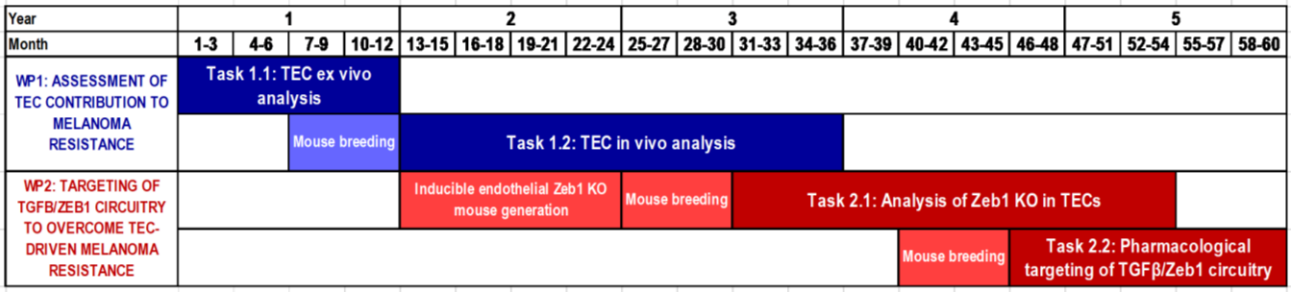
Task 2.2: Pharmacological targeting of TGFβ/Zeb1 circuitry (Mo: 40-60). To translate our studies to a preclinical setting, the therapeutic efficacy of small molecules interfering with TGFβ/Zeb1 circuitry combined with

COMBO will be evaluated together with Dr Nisticó. TGFβ/Zeb1 circuitry will be targeted with: TGFβ inhibitors (LY2157299); and Zeb1 indirect inhibitors (AA6; and MS275). Task 2.2 will be brought down into sub-tasks.
Subtask 2.2.1. In vitro experiments. The effect of the combination of PLX4720 and small molecules will be evaluated in vitro in human and mouse melanoma cells bearing BRAF mutation. Cells viability, expression of Zeb1-dependent signature, levels of secreting molecules and assay of trans-endothelial migration will be assessed. Data generated within this task will identify the most promising combination to adopt in Subtask 2.2.2.
Subtask 2.2.2. In vivo experiments. iEC^{tomato} mice bearing BRAF mutated melanoma will be treated with the selected small molecule showing the highest outcome (see Subtask 2.2.1) alone or in combination with COMBO. This combinatorial therapeutic approach will be tested for its ability to prevent or delay melanoma resistance to BRAFi and ICB therapy and the obtained data will be compared with effect of genetic deletion of Zeb1 into the endothelium (Task 2.1). Specifically, we will evaluate: 1) tumor outgrowth; 2) response to therapy; 3) entity of tumor-infiltrating leukocytes; 4) markers of immune activation/exhaustion, endothelial activation and EndMT; 5) Zeb1-dependent mediators (Task 2.1) by qRT-PCR; 6) levels of TGFβ and secreting molecules derived from Task 1.2. The results obtained will provide a new combinatorial therapeutic approach of clinical relevance to counteract the onset of melanoma resistance to BRAFi and ICB therapy (**Objective 5**).

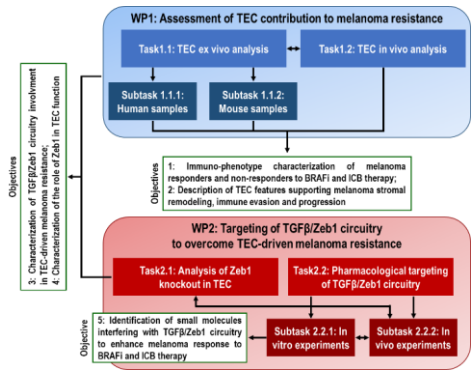
3.2 MILESTONES AND DELIVERABLES

WP	TASK	Milestones	Deliverables
WP1	TASK1.1	***M1)*** *Immuno-phenotype characterization of melanoma responders and non-responders to BRAFi and ICB therapy;* ***M2)*** *Assessment of TEC phenotype responsible of stromal remodeling, immune escape and melanoma progression.*	***D1)*** *Endothelial reporter mouse model.*
	TASK1.2	***M3)*** *Identification of molecular mediators of TEC plasticity and TEC-dependent immune evasion;* ***M4)*** *Identification of the percentage of CAF deriving from TEC;* ***M5)*** *Assessment of the molecular mechanism harnessing melanoma TEC anergy and EndMT.*	***D2)*** *List of secreted molecules and transcripts interfering with response to BRAFi and ICB therapy (TEC-driven signature of melanoma resistance to BRAFi and ICB therapy).*
WP2	TASK2.1	***M6)*** *Identification of Zeb1 role in TEC alterations supporting melanoma acquired resistance.*	***D3)*** *Conditional Zeb1 knock-out mouse.* ***D4)*** *Zeb1-dependent mediators of TEC-driven melanoma resistance to BRAFi and ICB therapy.*
	TASK2.2	***M7)*** *Identification of small molecules favoring response to BRAFi and Anti-PD1 therapy, delaying/preventing melanoma resistance.*	***D5)*** *List of small molecules interfering with TGFβ/Zeb1 circuitry with clinical relevance for melanoma patients.*

3.3 GANTT CHART



4. RESOURCES AND FEASIBILITY



Several factors lay the groundwork to successfully carry out the present proposal. Firstly, the state-of-the-art techniques for which appropriate expertise and instruments are available in our Institution at the CNR. Among major equipment: QS Series Real Time PCR (Applied Biosystems); QX-200 Droplet Digital PCR (Bio-Rad); Bioruptor Sonicator; Nanodrop; Confocal microscope; reverted-phase contrast-, immunofluorescence-, video time-lapse-, stereotactic-microscopes, flow cytometer, thermocycler, Instant Imager, VersaDoc 3000, Victor2 (fluorimeter, luminometer, ELISA reader), ELISA Readers/Washers, Seahorse Biosciences® XP technology, spectrophotometer, cryostat, microtome, vibratome. A cell culture facility (BSL1-2) and a cold room are available. Secondly, the outstanding collaborator network with heterogeneous and specific expertise (see Collaboration Letters), of whom we will avail ourselves. *Dr Sapino,* working at the Surgical Pathology of Candiolo Cancer Institute, and *Dr Peris,* working at Policlinico Gemelli, will give the opportunity to conduct an analysis on the status of melanoma endothelium in a cohort of melanoma patients stratified by disease stage and follow-up data. *Dr Bernardini*, pertaining to Department of Molecular Medicine at Sapienza University, will contribute to analyze leukocyte trafficking. *Dr Bussolino* (Department of Oncology - University of Turin), who will provide his longstanding background on vascular biology and his expertise on melanoma preclinical models, will actively participate with his team to all in vivo experiments making available to the PI the following Core Facilities: 1) Genomics; 2) Flow Cytometry Sorting Center; 3) Imaging; 4) Barriered animal facility. He will allow the PI access to the following equipment to achieve the described aims: 3 NGS platforms (2 Illumina MiSeq, 1 Illumina Next-Seq500), a C1 single-cell auto prep system for single-cell genomics (Fluidigm), a 96-capillary Sanger DNA Analyzer (ABI 3730), 1 robotic stations (Hamilton) and 2 QX200 Droplet Digital PCR systems; tissue culture facilities (BSL 1-3), confocal microscopes and video-lapse imaging devices equipped for STED, TIRF, FRET and FRAP (Leica, TCS SP5 X, TCS SP8 II, DMI6000 B), FACS Sorter (Moflo), Cytofluorimetries CyAN ADP. *Dr Chiani and Dr Gambadoro* will allow the access to the Transgenic Animals Facility of the CNR, crucial for the generation of the inducible endothelial Zeb1 knockout mouse (B6 -Zeb1$^{<tm1a(MFAG)Cnrm>}$ Tg(Cdh5-cre/ERT2)1Rha/Cnrm). This genetic mouse model will help to understand whether the absence of Zeb1 expression in the endothelium is able to favor melanoma response to therapy. The expertise of *Dr Nisticó*, head of Tumor Immunology and Immunotherapy Unit at Regina Elena National Cancer Institute, will represent a plus in the interpretation of our novel combinatorial therapy outcome and will support 3D scaffold model experiments. *Dr Bertinaria* (Dept. of Drug Science and Technology - University of Turin) will make available his expertise in designing and synthetizing compounds able to interfere with TGFβ/Zeb1 circuitry. Moreover, his background in drug science and technology will contribute to the interpretation of experimental evidences related to the pharmacological effects of our small molecules. Thus, the research network, our background and heterogeneous expertise, together with the described facilities and technical support, are valuable assets for the development of the proposed project and for its completion within a 5-year time frame. Lastly, the PI, *Dr Cencioni*, has a multi-disciplinary international scientific experience with a strong background in molecular biology applied to translational research. Her expertise in Zeb1 function, vascular biology, immunology and her technical skills related to trans-endothelial migration, in vivo experiments, RNASeq technology will help the supervision of the present proposal and will allow the accomplishment of the project goals (24,25,35,39,40).

5. RISK ASSESSMENT AND CONTIGENCY PLANS

WP1. The specific analysis of resistance to PLX4720 and Anti-PD1 treatment might limit the relevance of our study. For this reason, the identified mediators of TEC-dependent melanoma resistance will be matched in melanoma samples derived from patients developing resistance to other BRAFi (including dabrafenib and encorafenib), MAPKi, usually used in combination with BRAFi (like cobimetinib, trametinib and binimetinib), and ICB therapy based on CTLA4 antibody exploiting the collaboration with the Surgical Pathology of the Candiolo Cancer Institute and Policlinico Gemelli. To further increase the relevance of our studies, iEC^{tomato} mice will be sc injected with other melanoma cells lines bearing different mutational patters in association with BRAF mutation (Braf$^{V600E/wt}$ Cdkn2$^{-/-}$; Braf$^{V600E/wt}$ Pten$^{-/-}$ Cdkn2$^{-/-}$; Braf$^{V600E/wt}$ Pten$^{-/-}$ Cdkn2$^{-/-}$ Mc1r $^{e/e}$) and immunotherapy resistance will be evaluated also considering treatment with CTLA4 antibody.

WP2. TGFβ might cooperate with other cytokines to modulate TEC activity which favors resistance. The sequencing analysis and the secretome analysis will be exploited to evaluate the contribution of other signaling pathways to the onset of EC anergy and EndMT expanding our knowledge to different molecular circuitries involved in TEC-driven melanoma resistance. In case experimental data will highlight mediators of TEC-driven melanoma resistance not interconnected to TGFβ/Zeb1 circuitry, specific pharmacological inhibitors will be tested in Task 2.2. In case our results will highlight an essential role of Zeb1 in TEC-driven melanoma resistance, Zeb1 ChIPSeq analysis will be performed in isolated TECs. In the event that the strategy adopted to generate conditional Zeb1$^{iEC-/-}$ mouse will fail, despite the longstanding expertise of our collaborators, a different strategy based on CRISPR/Cas9 technology will be adopted. 3D scaffold experimental models will be exploited to recreate the complexity of tumor microenvironment and study the effects of drug combinations (41)**.** 3D scaffold models, generated by Dr Nisticó, will entail the co-culture of BRAF mutated melanoma cells, TECs, CAFs and monocytes and will represent a tool to test the most promising combination therapy. In case small molecules used will fail to efficiently target TGFβ/Zeb1 circuitry, we will exploit the expertise of Dr Bertinaria to design and test novel compounds aimed to target altered pathways identified alongside our study on melanoma resistance.

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PERSONNEL INVOLVED IN THE RESEARCH

Team Members

		1st year		2nd year		3rd year		4th year		5th year	
Role, name and affiliation	**C***	**Financial support**	**Effort**	**Financial support**	**Effort**	**Financial support**	**Effort**	**Financial support**	**Effort**	**Financial support**	**Effort**
Principal Investigator											
Cencioni Chiara (03/04/1982) Consiglio Nazionale delle Ricerche	**N**	**N**	**0,60**	**N**	**0,60**	**N**	**0,50**	**N**	**0,50**	**N**	**0,50**
Early stage researchers											
TBD	N	€ 28k	1,00	€ 28k	1,00	€ 28k	1,00	€ 28k	1,00	€ 28k	1,00
Experienced researchers											
Santoni Daniele (31/07/1971) Consiglio Nazionale delle Ricerche	N	N	0,00	N	0,20	N	0,30	N	0,30	N	0,20

Total Man/Year on project	**1,6**		**1,8**		**1,8**		**1,8**		**1,7**

C* = Clinician

Collaborators

Name and affiliation	Clinician
Bernardini Giovanni Università degli Studi di Roma "La Sapienza"	N
Bertinaria Massimo Università degli Studi di Torino	N
Bussolino Federico Università degli Studi di Torino	N
Chiani Francesco Consiglio Nazionale delle Ricerche	N
Nisticó Paola Regina Elena National Cancer Institute	N
Peris Ketty Policlinico Universitario "Agostino Gemelli"	Y
Sapino Anna Istituto di Candiolo - Fondazione del Piemonte per l'Oncologia (FPO) - I.R.C.C.S.	N

Supporting documentation available in:

Addendum A - PERSONNEL INVOLVED IN THE RESEARCH - CURRICULUM VITAE

Collaboration letters are required from Collaborators only (individual researchers and companies). CVs are required for personnel as specified in the Call.

Supporting documentation available in:

Addendum B - PERSONNEL INVOLVED IN THE RESEARCH - LETTERS OF COLLABORATION

The proposed experimental program will be performed at the CNR- Istituto di Analisi dei Sistemi ed Informatica "Antonio Ruberti" (IASI) (Institute for Systems Analysis and Computer Science) in the secondary institute site in Rome dislocated into two units at Policlinico Gemelli and at the Medical School of Catholic University of Sacred Heart (UCSC), which research activities focus on physiopathology, metabolism, oncology, and immunology to uncover novel molecular and cellular mechanisms contributing to the development of human pathologies. The laboratories located there are fully equipped for standard molecular/cell biology experiments. In addition, the PI will have full access to the core facilities and technological platforms of the Department of Oncology at University of Turin, headed by Prof Federico Bussolino, which are necessary to perform the suggested in vivo experiments and NGS sequencing of melanoma endothelial cells.

Dr Chiara Cencioni, PI. I will supervise and coordinate the whole study. I will supervise the postdoctoral fellow (TBD) in experiments aimed at the characterization of the role of tumor associated endothelial cells in the onset of melanoma resistance to BRAF inhibition and immunotherapy. I will coordinate/perform analysis on Zeb1 molecular functions and the entire research network personally interacting with all collaborators listed in the present document. Moreover, I will conceive experimental strategies to reach aims described in the project proposal and write abstracts for international meetings and manuscripts to be submitted for publication related to the results of the present proposal.

Post-doctoral fellow (TBD) will perform most of in vivo experiments taking care of mouse breeding to maintain genetic engineered mouse model colonies and related genotyping. He/she will subcutaneously inject syngeneic BRAF mutant mouse melanoma cells in 1) iEC^{tomato}; and 2) $Zeb1^{iEC-/-}$ mice and administered Anti-PD1; PLX4720; TGFβ/Zeb1 circuitry inhibitors. Then, he/she will take care of the subsequent immunofluorescence and immunohistochemistry analyses to dissect the role of tumor endothelial cell in melanoma resistance. Moreover, he/she will perform molecular techniques.

Dr Daniele Santoni, working at the CNR-IASI will take care of all bioinformatics analyses necessary for the interpretation of RNA-Seq data deriving from the experiments planned in the present proposal. Moreover, he will help to define the Zeb1 network contributing to the tumor endothelial cell-driven melanoma resistance.

Prof Anna Sapino, Director of the Surgical Pathology at Candiolo Cancer Institute will provide melanoma samples derived from a cohort of patients diagnosed and followed-up at the cancer institute. A member of her team, an experienced dermatopathologist, will provide data related to the histopathologic analysis of the cohort of melanoma patient samples.

Prof Ketty Peris, a medical oncologist pertaining to the Medical School of Catholic University of Sacred Heart (UCSC) and Policlinico Gemelli will provide melanoma samples derived from a cohort of patients diagnosed and followed-up at her Unit and will support the interpretation of the experimental data on human melanoma samples matching them with clinical and histopathologic data.

Prof Federico Bussolino, head of the Department of Oncology at University of Turin will provide samples derived from mouse melanoma models upon treatment with PLX4720, PD1 antibody and both in combination. Members of his group will be actively involved in all in vivo experiments. Moreover, he will give access to the PI to the facilities of his institute for NGS-Sequencing.

Dr Paola Nisticó, head of Tumor Immunology and Immunotherapy Unit at Regina Elena National Cancer Institute, will provide her expertise in immuno-oncology and will support all the experiments aimed at studying interactions among tumor cells, CAF and immune cells through the establishment of 3D co-cultures. She will help designing novel treatment in combination with immunotherapy able to overcome melanoma therapy resistance.

Dr Francesco Chiani and Dr Alessia Gambadoro, working at the transgenic mouse facility of CNR, will generate the inducible endothelial Zeb1 KO mouse, fundamental to carry on the study of Zeb1 role in the endothelium of melanoma tissue during the onset of resistance to BRAF inhibitor and immunotherapy.

Prof Massimo Bertinaria, working at the Department of Science and Technology at the University of Turin will provide AA6 molecule and will make available his expertise in designing and synthetizing compounds able to interfere with the TFGβ/Zeb1 circuitry.

Relevant reference for each unit.

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BUDGET FORM AND JUSTIFICATIONS

	1st year	2nd year	3rd year	4th year	5th year	Total
Direct Research costs						
- Consumables and supplies	51.600,00 €	18.050,00 €	28.995,00 €	42.425,00 €	45.750,00 €	**186.820,00 €**
- Small bench instrumentation	0,00 €	0,00 €	0,00 €	0,00 €	0,00 €	**0,00 €**
- Services	0,00 €	30.000,00 €	12.000,00 €	0,00 €	0,00 €	**42.000,00 €**
- Maintenance contracts	2.074,80 €	5.810,20 €	7.995,20 €	6.566,40 €	3.241,40 €	**25.688,00 €**
- Publication costs	0,00 €	0,00 €	3.000,00 €	3.000,00 €	3.000,00 €	**9.000,00 €**
- Meetings and travel costs	950,00 €	750,00 €	2.650,00 €	2.650,00 €	2.650,00 €	**9.650,00 €**
Personnel costs	28.000,00 €	28.000,00 €	28.000,00 €	28.000,00 €	28.000,00 €	**140.000,00 €**
PI salary	0,00 €	0,00 €	0,00 €	0,00 €	0,00 €	**0,00 €**
Indirect costs *(10,0%)*	8.262,48 €	8.261,02 €	8.264,02 €	8.264,14 €	8.264,14 €	**41.315,80 €**
SUBTOTAL	**90.887,28 €**	**90.871,22 €**	**90.904,22 €**	**90.905,54 €**	**90.905,54 €**	**454.473,80 €**
Overheads *(10,0%)*	9.088,73 €	9.087,12 €	9.090,42 €	9.090,55 €	9.090,55 €	**45.447,37 €**
TOTAL	**99.976,01 €**	**99.958,34 €**	**99.994,64 €**	**99.996,09 €**	**99.996,09 €**	**499.921,17 €**

By signing the application, the Legal Representative acknowledges the budget requested, including “Indirect costs” and “Overheads”, in compliance with the guidelines of the Call.

1ST YEAR JUSTIFICATION NOTES

Consumables (tot 51600€):

-22500 €: 50 antibodies to study tumor cell infiltration, to evaluate endothelial cell anergy and to analyse endothelial to mesenchymal transition. Approximate cost for each antibody 450 € (List of necessary antibody for: CD45; CD3; CD4; CD8; gdTCR; CD19; CD11b; Ly6G; Ly6C; CD11b; F4/80; CD11c; MHCII; iNOS; CD206; NK1.1; NKp46; CD49b; CD27; Foxp3; PD-1; CTLA4; LAG3; 2B4; TIM3; BTLA; TIGIT; Sca1; CD69; CD25; 4-1BB; TCF1; KLRG1; P-selectin; E-selectin; ICAM1; VCAM1; ICAM2; VE-cadherin; JAMs; ESAM; CD31; CD99; CD99L2; JAMA; Zeb1; TWIST; Vimentin; aSMA; SM22a; FAP; Vinculin).

-5000 €: Reagents for DNA purification kit for genotyping mice derived from the mating between Cdh5-CreERT2 mice and R26Rosa-lox-Stop-lox-tdTomato-hrLuc mice and reagents for enzymes, PCR and oligos.

-7400 €: Reagents for immunofluorescence analyses. They include solvents, buffers and chemicals for confocal microscopy analysis and flow cytometry analysis, mounting medium, Immunostaining Pen (PAP Pen), secondary antibodies (300 € each with different fluorochrome and against different host for multi-staining images to be used for Confocal analysis and flow cytometry analysis), DAPI for nucleus staining.

-11500 €: Disposable and plastics and glassware. They include supplies for tissue culture, including disposable tissue culture pipets; tips; sterile aerosol barrier pipet tips; multi-well plates, including ELISA plates; 50 ml, 15 ml, 5 ml, 2 ml, 1.5 ml, 0.5 ml, 0.2 ml tubes; scrapers; flow cytometry tubes; and filters; multi-channel pipet basins, sterile filtration units, syringes, needles, everything is need for histology analyses (Microscope Slides; Cover Slip; Slide Boxes, Holders and Trays; Permanox and Polystyrene Microscope Slides; Polysine Microscope Adhesion Slide).

-1600 €: for at least 2 TGF-beta ELISA KIT (800 € each)

-3600 €: Reagents for immunohistochemistry analyses. They include wash solutions, stains, blocking agents, detection, mounting solutions, blocking solutions, streptavidin-HRP reagents, fixation/permeabilization/clearing Reagents, unmasking fluids.

Maintenance contracts (tot 2074.8)

-The proposal requires 2 different GEMM colonies: Cdh5-CreERT2 mice and R26Rosa-lox-Stop-lox-tdTomato-hrLuc mice. According the Italian law, every cage can hold up to 5 mice. The cost per day per cage is 1.9 €. Cost estimated for breeding and iEC dtomato mouse colony maintenance (6 cages up to 6 months).

Meeting and travel costs (tot 950 €)

-The first year experiments will be conducted between Rome and Turin. The PI estimated travel costs to supervise the activity at the Department of Oncology in Turin attending meeting to plan activities and discuss data.

Personnel efforts/costs (28000 €)

-This is the cost required for an early stage researcher at 100% time on the project according CNR rules.

Indirect costs and Overheads have been calculated according the policy of CNR.

2ND YEAR JUSTIFICATION NOTES

Consumables (tot 18050 €):

-2306.8 €: WB reagents including laemmi lysis buffer, running buffers, dual colour protein ladder, pre-casted gels, nitrocellulose trans-blot transfer packs, secondary antibodies, ECL kit, milk blocking powder.

-3700 €: RNA and qRT-PCR reagents including AS1460 Maxwell RSC miRNA Tissue Kit (322 €/kit for 48 samples), reverse transcriptase (1200 €), sybr green (1900 €), Oligo synthesis primers (20€ each) and Taqman probes (346 € each).

-800 €: 1 ELISA kit for TGFbeta quatification

-2500 €: Disposable and plastics including supplies for tissue culture, including disposable tissue culture pipets; tips; sterile aerosol barrier pipet tips; multi-well plates, including ELISA plates; 50 ml, 15 ml, 5 ml, 2 ml, 1.5 ml, 0.5 ml, 0.2 ml tubes; scrapers.

-5763.2 €: Secretome analysis performed by a Luminex xMAP multiplex bead-based assay approach, using antibody panels for Cancer Biomarkers (R&D Systems 10-plex 2012,8 €: Fas Ligand; basic FGF; HGF; IL-6; CXCL10; Leptin; Osteopontin; Prolactin; TNFα; VEGF) and for Immuno-Oncology Checkpoint (R&D Systems 20-plex 3750,4 €: GM-CSF; Granzyme B; IFNγ; IL-1α; IL-1β; IL-10; IL-12 p70; IL-13; IL-17A; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; MCP-2/CCL8; MCP-3/CCL7; MICA; MIP-1α/CCL3; MIP-1β/CCL4)

-2980 €: Drugs for mouse treatment including PLX4720 and anti-PD1

Maintenance contracts (tot 5810.2 €)

-The cost per day per cage is 1.9 €. Cost estimated for mouse colony maintenance and experiment in a time course up to 6 weeks.

Service (tot 30000 €)

-Cost for the strategy proposed to generate Inducible endothelial Zeb1 knock-out (KO) mice at the Transgenic mouse facility of CNR by Dr Chiani and Dr Gambadoro based on targeted homologous recombination in murine embryonic stem cells (mESCs).

Meeting and travel costs (tot 750 €)

-Travel costs to attend meeting at Department of Oncology in Turin to plan the activities and discuss data.

Personnel efforts/costs (28000 €)

-This is the cost required for an early stage researcher at 100% time on the project according CNR rules.

Indirect costs and Overheads have been calculated according the policy of CNR.

3RD YEAR JUSTIFICATION NOTES

Consumables (tot 28995 €):

-3650 €: WB reagents including laemmi lysis buffer, running buffers, dual colour protein ladder, pre-casted gels, nitrocellulose trans-blot transfer packs, secondary antibodies, ECL kit, milk blocking powder.

-3000 €: Reagents for DNA purification kit for genotyping mice derived from the mating between Cdh5-CreERT2 mice and B6 -Zeb1< tm1a(MFAG_CC)Cnrm > Tg(Cdh5-cre/ERT2)1Rha/Cnrm and reagents for enzymes, PCR and oligos.

-6550 €: RNA and qRT-PCR reagents including AS1460 Maxwell RSC miRNA Tissue Kit (322 €/kit for 48 samples), reverse transcriptase (1200 €), sybr green (1900 €), Oligo synthesis primers (20€ each) and Taqman probes (346 € each).

-7400 €: Disposable and plastics including supplies for tissue culture, including disposable tissue culture pipets; tips; sterile aerosol barrier pipet tips; multi-well plates, including ELISA plates; 50 ml, 15 ml, 5 ml, 2 ml, 1.5 ml, 0.5 ml, 0.2 ml tubes; scrapers.

-4404 €: Cytokine and GF to be supplemented to media for evaluation of TEC function.

-3991 €: Reagents for isolation of tumor endothelial cells purchased by Miltenyi including columns (25 columns 221 €), beads (100 preparations 697 €), separation buffer (146 €) and tumor dissociation kit (560 €).

Maintenance contracts (tot 7995.2 €)

-The cost per day per cage is 1.9 €. Cost estimated for mouse colony maintenance and experiment in a time course up to 6 weeks.

Service (tot 12000 €)

-NGS Sequencing performed at the Genomic Facility of the Department of Oncology of the University of Turin. 12000 € cost estimated for RNASeq (500 €/sample).

Meeting and travel costs (2650 €)

-Conference fee and travel costs to reach the venue. Attendance to an international meeting to share data acquired alongside the project.

Publication (3000 €)

-Publication fee for 1 manuscript.

Personnel efforts/costs (28000 €)

-This is the cost required for an early stage researcher at 100% time on the project according CNR rules.

Indirect costs and Overheads have been calculated according the policy of CNR.

4TH YEAR JUSTIFICATION NOTES

Consumables (tot 42425 €):

-8435 €: RNA and qRT-PCR reagents including AS1460 Maxwell RSC miRNA Tissue Kit (322 €/kit for 48 samples), reverse transcriptase (1200 €/each), sybr green (1900 €/each), Oligo synthesis primers (20€ each) and Taqman probes (346 € each).

-3515.8 €: WB reagents including laemmi lysis buffer, running buffers, dual colour protein ladder, pre-casted gels, nitrocellulose trans-blot transfer packs, secondary antibodies, ECL kit, milk blocking powder.

-8000 €: Disposable and plastics and glassware. They include supplies for tissue culture, including disposable tissue culture pipets; tips; sterile aerosol barrier pipet tips; multi-well plates, including ELISA plates; 50 ml, 15 ml, 5 ml, 2 ml, 1.5 ml, 0.5 ml, 0.2 ml tubes; scrapers; flow cytometry tubes; and filters; multi-channel pipet basins, sterile filtration units, syringes, needles, everything is need for histology analyses (Microscope Slides; Cover Slip; Slide Boxes, Holders and Trays; Permanox and Polystyrene Microscope Slides; Polysine Microscope Adhesion Slide).

-4631 €: Reagents for isolation of tumor endothelial cells purchased by Miltenyi including columns (25 columns 221 €), beads (100 preparations 697 €), separation buffer (146 €) and tumor dissociation kit (560 €).
-2980 €: Drugs for mouse treatment including PLX4720 and anti-PD1
-4000 €: 10 antibodies to evaluate endothelial cell anergy and to analyse endothelial to mesenchymal transition. Approximate cost for each antibody 400 € (E-selectin; ICAM1; VCAM1; ICAM2; VE-cadherin; CD31; Zeb1; Vimentin; aSMA; FAP).
-1600 €: for at least 2 TGF-beta ELISA KIT (800 € each)
-5763.2 €: Secretome analysis performed by a Luminex xMAP multiplex bead-based assay approach, using antibody panels for Cancer Biomarkers (R&D Systems 10-plex 2012,8 €: Fas Ligand; basic FGF; HGF; IL-6; CXCL10; Leptin; Osteopontin; Prolactin; TNFα; VEGF) and for Immuno-Oncology Checkpoint (R&D Systems 20-plex 3750,4 €: GM-CSF; Granzyme B; IFNγ; IL-1α; IL-1β; IL-10; IL-12 p70; IL-13; IL-17A; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; MCP-2/CCL8; MCP-3/CCL7; MICA; MIP-1α/CCL3; MIP-1β/CCL4)
-3500 €: Reagents for immunofluorescence and immunohistochemistry analyses including solvents, buffers and chemicals for confocal microscopy analysis and flow cytometry analysis, mounting medium, Immunostaining Pen (PAP Pen), secondary antibodies (300 € each with different fluorochrome and against different host for multi-staining images to be used for Confocal analysis and flow cytometry analysis), DAPI, wash solutions, stains, blocking agents, detection, blocking solutions, streptavidin-HRP reagents, fixation/permeabilization/clearing reagents, unmasking fluids.
Maintenance contracts (6566.4 €)
-The cost per day per cage is 1.9 €. Cost estimated for mouse colony maintenance and experiment in a time course up to 6 weeks.
Meeting and travel costs (2650 €)
-Conference fee and travel costs to reach the venue. Attendance to an international meeting to share data acquired alongside the project.
Publication (3000 €)
-Publication fee for 1 manuscript.
Personnel efforts/costs (28000 €)
-This is the cost required for an early stage researcher at 100% time on the project according CNR rules.
Indirect costs and Overheads have been calculated according the policy of CNR.

5TH YEAR JUSTIFICATION NOTES

Consumables (tot 45750 €)
-9410 €: RNA and qRT-PCR reagents including AS1460 Maxwell RSC miRNA Tissue Kit (322 €/kit for 48 samples), reverse transcriptase (1200 €/each), sybr green (1900 €/each), Oligo synthesis primers (20€ each) and Taqman probes (346 € each).
-5500 €: WB reagents including laemmi lysis buffer, running buffers, dual colour protein ladder, pre-casted gels, nitrocellulose trans-blot transfer packs, secondary antibodies, ECL kit, milk blocking powder.
-8000 €: Disposable and plastics and glassware. They include supplies for tissue culture, including disposable tissue culture pipets; tips; sterile aerosol barrier pipet tips; multi-well plates, including ELISA plates; 50 ml, 15 ml, 5 ml, 2 ml, 1.5 ml, 0.5 ml, 0.2 ml tubes; scrapers; flow cytometry tubes; and filters; multi-channel pipet basins, sterile filtration units, syringes, needles, everything is need for histology analyses (Microscope Slides; Cover Slip; Slide Boxes, Holders and Trays; Permanox and Polystyrene Microscope Slides; Polysine Microscope Adhesion Slide).
-1991 €: Reagents for isolation of tumor endothelial cells purchased by Miltenyi including columns (25 columns 221 €), beads (100 preparations 697 €), separation buffer (146 €) and tumor dissociation kit (560 €).
-7249 €: Drugs for mouse treatment including PLX4720 and anti-PD1
-2500 €: 5 antibodies to evaluate endothelial cell anergy and to analyse endothelial to mesenchymal transition. Approximate cost for each antibody 500 € (E-selectin; CD31; Zeb1; aSMA; FAP).
-1600 €: for at least 2 TGF-beta ELISA KIT (800 € each)
-2500 €: Reagents for immunofluorescence analyses including solvents, buffers and chemicals for confocal microscopy analysis and flow cytometry analysis, mounting medium, Immunostaining Pen (PAP Pen), secondary antibodies (300 € each with different fluorochrome and against different host for multi-staining images to be used for Confocal analysis and flow cytometry analysis), DAPI for nucleus staining.
-4500 €: Reagents for DNA purification kit for genotyping mice derived from the mating between Cdh5-CreERT2 mice and R26Rosa-lox-Stop-lox-tdTomato-hrLuc mice and reagents for enzymes, PCR and oligos.

-2500 €: Cytokine and GF to be supplemented to media for evaluation of TEC function.

Maintenance contracts (tot 3241.4 €)

-The cost per day per cage is 1.9 €. Cost estimated for mouse colony maintenance and experiment in a time course up to 6 weeks.

Meeting and travel costs (2650 €)

-Conference fee and travel costs to reach the venue. Attendance to an international meeting to share data acquired alongside the project.

Publication (3000 €)

-Publication fee for 1 manuscript.

Personnel efforts/costs (28000 €)

-This is the cost required for an early stage researcher at 100% time on the project according CNR rules.

Indirect costs and Overheads have been calculated according the policy of CNR.

DISCLOSURE OF FINANCIAL CONFLICTS OF INTEREST

According to the AIRC policy, a financial conflict of interest includes a financial association or relationship that could influence the objectivity, integrity, or interpretation of a research activity.
Such conflicts of interest include relationships with corporations whose products or services are related to the subject matter of the research proposal.
These relations include employment, substantive ownership of stock (> 5% of shares in a company), membership on a standing advisory council or committee, service on the board of directors, or public association with the company or its products.
Other areas of conflict of interest could include receiving consulting fees (> 10.000 € per year from a company), patent filings, serving as a paid spokesperson, or providing services in exchange for honoraria.

Disclosure statement:

- [x] «I do not have financial conflicts of interests, as defined in the AIRC policy»
- [ ] «I do have financial conflicts of interests, as described in the AIRC policy, which are not related to the present proposal»
- [ ] «I do have financial conflicts of interests, as described in the AIRC policy, which may be perceived as related to the present proposal»

BIOGRAPHICAL SKETCH

PERSONAL DATA OF THE PI	
Surname Cencioni	**Name** Chiara
Position Junior CNR Researcher	**Date of birth** 03/04/1982

EDUCATION AND TRAINING (DEGREES)					
Duration (from/to)	**Degree and Field of study**	**Institution**	**Supervisor/ Mentor**	**City**	**Country**
Nov 2007/ Oct 2010	PhD - Immunological Sciences	Università degli Studi di Roma "La Sapienza"	Santoni Angela	Roma	Italy
Nov 2004/ Dec 2006	Master Degree - Molecular, cellular and medical biotechnologies	Università degli Studi di Roma "La Sapienza"	Santoni Angela	Roma	Italy
Nov 2001/ Oct 2004	Bachelor Degree - Biotechnologies	Università degli Studi di Roma "La Sapienza"	Paolini Rossella	Roma	Italy

RESEARCH AND PROFESSIONAL EXPERIENCE (INCLUDES POST-DOCTORAL TRAINING)					
Duration (from/to)	**Position**	**Institution**	**Supervisor/ Mentor**	**City**	**Country**
Sep 2019/ Present	Junior CNR researcher	Institute for Systems Analysis and Computer Science (IASI) - CNR	-	Roma	Italy
Dec 2016/ Sep 2019	Junior CNR researcher	Institute of cellular biology and neurobiology (IBCN) - CNR	-	Roma	Italy
Aug 2012/ Dec 2016	Fixed term researcher (Wissenschaftlicher Mitarbeiterin)	Goethe University	Gaetano Carlo	Frankfurt am Main	Germany
Nov 2010/ Jun 2012	Postdoctoral fellow	Centro Cardiologico S.p.A. Fondazione Monzino I.R.C.C.S.	Pompilio Giulio	Milano	Italy
Nov 2007/ Oct 2010	PhD student	Università degli Studi di Roma "La Sapienza"	Santoni Angela	Roma	Italy
Jan 2007/ Oct 2007	Post graduate fellow	Istituto Dermopatico dell´Immacolata - IRCCS	Napolitano Monica	Roma	Italy

PARTICIPATION TO CONFERENCES				
Date	**Type of Contribution**	**Conference**	**Title**	**City - Country**
12 Nov 2016	Oral presentation	American Heart Association Scientific Session	Identification of Long non coding RNAs associated to mesoderm differentiation and cardiovascular commitment of early precursors by naive mouse embryonic st	New Orleans - United States of America
12 Sep 2016	Poster	German Stem Cell Network	Identification of early cardiovascula precursors in naive mouse embryonic stem cells	Frankfurt am Main - Germany
20 Oct 2015	Oral presentation	German-Italian centre for European excellence	Nitric oxide synthesis and Zeb1 transcription factor inactivation characterize an early mesoendoderm precursor population in mouse embryonic stem cells	Menaggio - Italy

AWARDS		
Date	**Award**	**Awarding Body**

15 Nov 2016	BCVS International Travel Award	The American Heart Association Council on Basic Cardiovascular Sciences
11 Sep 2015	Best poster at GSCN meeeting 2015 in Frankfurt am Main (Germany)	German Stem Cell Network sponsored by PeproTech

PI TRACK RECORD SUMMARY (2015-2020)

Principal Investigator's Full Name Cencioni Chiara

Total number of papers	**16**
Total number of papers with IF	**16**
Total IF	**114,746**
Average IF (of papers with IF)	**7,2**
Number of papers as First, Last, or Corresponding Author (all journals)	**10**
Number of papers as First, Last, or Corresponding Author (journals with IF)	**10**
Active IF	**84,091**
Average Active IF	**8,4**
Number of papers with acknowledgement to AIRC	**5**

This track record summary is based on the publications by the PI in the last five years (or more, depending on whether she/he has had research interruptions longer than 12 months). The publications are listed in the following page(s).
The applicant certifies that all papers have been carefully checked and correctly flagged for authorship.

PUBLICATIONS OF THE PI

The publications listed below are from the last five years, unless the applicant has had research interruptions longer than 12 months.

For each full year of research interruption the time range for papers listed in the application includes one additional year.

Title, list of authors and complete reference of all publications from 2015 to 2020

FA/CoF = First Author/Co-First Author

LA/CoL/CA/CC = Last Author/Co-Last Author/Corresponding Author/Co-Corresponding Author

Ackn. = Acknowledgement

Principal Investigator's Full Name: Cencioni Chiara

ORCID: 0000-0001-6284-539X

Publication	IF	FA/CoF	LA/CoL/CA/CC	Ackn.
Aging Triggers H3K27 Trimethylation Hoarding in the Chromatin of Nothobranchius furzeri Skeletal Muscle. **Cencioni C**, Heid J, Krepelova A, Rasa SMM, Kuenne C, Guenther S, Baumgart M, Cellerino A, Neri F, Spallotta F, Gaetano C **CELLS-BASEL** 2019 09; 8:	5,656	X		
Dissecting cytosine methylation mechanics of dysmetabolism. **Cencioni C**, Gaetano C, Spallotta F **AGING-US** 2019 01; 11: 837-838	5,515	X		
Fibroblasts in Nodular Sclerosing Classical Hodgkin Lymphoma Are Defined by a Specific Phenotype and Protect Tumor Cells from Brentuximab-Vedotin Induced Injury. Bankov K, Döring C, Ustaszewski A, Giefing M, Herling M, **Cencioni C**, Spallotta F, Gaetano C, Küppers R, Hansmann ML, Hartmann S **CANCERS** 2019 Oct; 11:	6,162			
P300/CBP-associated factor regulates transcription and function of isocitrate dehydrogenase 2 during muscle differentiation. Savoia M, **Cencioni C**, Mori M, Atlante S, Zaccagnini G, Devanna P, Di Marcotullio L, Botta B, Martelli F, Zeiher AM, Pontecorvi A, Farsetti A, Spallotta F, Gaetano C **FASEB J** 2019 Mar; 33: 4107-4123	5,391			AIRC
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Stable Oxidative Cytosine Modifications Accumulate in Cardiac Mesenchymal Cells From Type2 Diabetes Patients: Rescue by α-Ketoglutarate and TET-TDG Functional Reactivation. Spallotta F, **Cencioni C**, Atlante S, Garella D, Cocco M, Mori M, Mastrocola R, Kuenne C, Guenther S, Nanni S, Azzimato V, Zukunft S, Kornberger A, Sürün D, Schnütgen F, von Melchner H, Di Stilo A, Aragno M, Braspenning M, van Criekinge W, De Blasio MJ, Ritchie RH, Zaccagnini G, Martelli F, Farsetti A, Fleming I, Braun T, Beiras-Fernandez A, Botta B, Collino M, Bertinaria M, Zeiher AM, Gaetano C **CIRC RES** 2018 01; 122: 31-46	15,862	X		
Structural and biological characterization of new hybrid drugs joining an HDAC inhibitor to different NO-donors. Atlante S, Chegaev K, **Cencioni C**, Guglielmo S, Marini E, Borretto E, Gaetano C, Fruttero R, Spallotta F, Lazzarato L **EUR J MED CHEM** 2018 Jan; 144: 612-625	4,833			
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Age-dependent increase of oxidative stress regulates microRNA-29 family preserving cardiac health. Heid J, **Cencioni C**, Ripa R, Baumgart M, Atlante S, Milano G, Scopece A, Kuenne C, Guenther S, Azzimato V, Farsetti A, Rossi G, Braun T, Pompilio G, Martelli F, Zeiher AM, Cellerino A, Gaetano C, Spallotta F **SCI REP-UK** 2017 12; 7: 16839	4,011	X		
Dark Side of the Deep Heart. **Cencioni C**, Spallotta F, Gaetano C **CIRC-CARDIOVASC GENE** 2017 06; 10:	4,897	X		
Deciphering Histone Code Enigmas Sheds New Light on Cardiac Regeneration. **Cencioni C**, Spallotta F, Farsetti A, Zeiher AM, Gaetano C **CIRC RES** 2017 04; 120: 1370-1372	15,862	X		
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The double life of cardiac mesenchymal cells: Epimetabolic sensors and therapeutic assets for heart regeneration. **Cencioni C**, Atlante S, Savoia M, Martelli F, Farsetti A, Capogrossi MC, Zeiher AM, Gaetano C, Spallotta F **PHARMACOL THERAPEUT** 2017 03; 171: 43-55	9,396	X		

1,4-Dihydropyridines Active on the SIRT1/AMPK Pathway Ameliorate Skin Repair and Mitochondrial Function and Exhibit Inhibition of Proliferation in Cancer Cells. Valente S, Mellini P, Spallotta F, Carafa V, Nebbioso A, Polletta L, Carnevale I, Saladini S, Trisciuoglio D, Gabellini C, Tardugno M, Zwergel C, **Cencioni C**, Atlante S, Moniot S, Steegborn C, Budriesi R, Tafani M, Del Bufalo D, Altucci L, Gaetano C, Mai A **J MED CHEM** 2016 Feb; 59: 1471-91	6,054			AIRC
Design and synthesis of N-benzoyl amino acid derivatives as DNA methylation inhibitors. Garella D, Atlante S, Borretto E, Cocco M, Giorgis M, Costale A, Stevanato L, Miglio G, **Cencioni C**, Fernández-de Gortari E, Medina-Franco JL, Spallotta F, Gaetano C, Bertinaria M **CHEM BIOL DRUG DES** 2016 11; 88: 664-676	2,256			
Sirtuin function in aging heart and vessels. **Cencioni C**, Spallotta F, Mai A, Martelli F, Farsetti A, Zeiher AM, Gaetano C **J MOL CELL CARDIOL** 2015 Jun; 83: 55-61	5,055	X		

Supporting documentation provided by the applicant (e.g. authorship certifications, journal's letters of acceptance for papers in press) is available to reviewers upon request to the AIRC staff.

BIO-ETHICAL REQUIREMENTS

Research on humans

Does the research plan include clinical trials with patients and/or healthy volunteers, or involve the use of human biological samples, genetic material or data collection?

☑ YES

☐ NO

Research on animals

Does the proposed research involve animal experimentation?

☑ YES

☐ NO

The applicant declares that the principles of the three Rs (Replacement, Reduction, Refinement) have been implemented in the research plan. The 3R document is available to the reviewers upon request.

☑ Animal experimentation: Principles of the 3Rs

Statement on bio-ethical requirements

• No research with humans or animals will be undertaken in the absence of the necessary authorizations; grant money cannot be used to cover costs associated with studies with humans or animals if the studies have not been authorized by the competent authorities.

• AIRC reserves the right to check the compliance with the bio-ethical requirements. To this aim, a copy of the authorizations must be made available to AIRC upon request at any time throughout the duration of the project and up to 10 years after the project is concluded.

• In case the competent authorities do not approve the proposed human or animals studies, the PI must promptly notify AIRC and devise an alternative research plan.

• Should there be substantial modifications in the research plan which require research on humans or animal experimentation not foreseen in this application, the PI must detail them in the grant renewal requests.

• Any proposed modifications of the research plan will be subject to scientific evaluation, to make sure that the project is still scientifically sound and competitive. Project continuation and renewals of the grant will be contingent on a positive evaluation of the proposed modifications.

• AIRC is not responsible and does not accept any liability for studies regarding research with humans and research with animals.

☑ I have read and agree with the bio-ethical requirements mandated by AIRC.

By signing the application, the PI and the Legal Representative of the Hosting Institution certify that they will comply with AIRC terms and conditions regarding research with humans and research with animals.

I am affiliated with the Hosting Institution at the application submission deadline.

☑ YES ☐ NO

By signing the application, the Legal Representative of the Hosting Institution confirms that the PI is affiliated with the Hosting Institution and declares that, in case the application is funded, the PI will be allowed to remain affiliated with the Hosting Institution for the entire duration of the grant in order to carry out the research project.

The Hosting Institution is the only institution I am affiliated with.

☑ YES ☐ NO

AIRC recognizes that scientific research conducted to the highest standards of integrity is essential to ensure high quality, reproducible and trustworthy results. AIRC supports the principles and guidelines on research integrity set out by the All European Academies federation (European Code of Conduct for Research Integrity), and trusts that the scientific endeavors of researchers funded by AIRC are driven by common values of rigor, honesty and responsibility.

Based on these premises, personnel (scientific and administrative staff) involved in a research project funded by AIRC is expected to comply with ethical principles of good scientific practice and engage in honest behavior. In particular, research centers where scientists conduct AIRC-funded research must:

1. Promote principles of rigor, honesty and integrity in research, e.g. by establishing and implementing rules of good scientific practice; setting up guidelines to properly manage, store and archive original data and images; mentoring junior scientists and offering them training opportunities on responsible conduct of research.

2. Have in place a formal, written policy for preventing and dealing with scientific misconduct. AIRC reserves the right to request a copy of such document. Scientific misconduct, i.e. a deliberately dishonest behavior against standards of integrity, rigor and good scientific practice, includes but is not limited to: fabrication, falsification, omission or improper representation of scientific results; plagiarism; violation of ethical standards and protocols for human subject or animal research. Scientific misconduct does not include honest, unintentional errors. The policy should describe: the procedures and timescales for enquiries and investigations; possible sanctions in case of proven misconduct; how an appeal can be made; the procedures to ensure the confidentiality of the investigation and of all parties involved.

3. Investigate allegations of scientific misconduct. In case of allegations involving researchers funded by AIRC, the institution should notify AIRC and keep it informed. AIRC reserves the right to suspend the grant during the investigation and, in case the allegations are upheld, to impose sanctions that may include: the termination of the grant; the obligation to return grant money to AIRC; the ineligibility to apply to AIRC grants; the exclusion from AIRC review panels and other bodies.

☑ By signing the application, the PI and the Legal Representative of the Hosting Institution certify that they will comply with ethical principles of good scientific practice and engage in honest behaviour, as described in this policy.

ACCESS TO CINECA RESOURCES

In agreement with AIRC, the Consorzio Interuniversitario CINECA is committed to provide free access to its High Performance Computing services and infrastructure to applicants who request them through this form and whose application gets funded.
Details on the CINECA bioinformatics environment can be found at: http://www.hpc.cineca.it/content/hpc-bioinformatics.

Do you plan to analyze NGS experiments with CINECA automated pipelines for NGS analysis? (http://www.hpc.cineca.it/content/hpc-bioinformatics)?

- [ ] YES
- [x] NO

Do you request to use CINECA High Performance Computing resources for the current proposal?
If you checked “YES” to the previous question, please complete this section as well.

- [ ] YES
- [x] NO

- [ ] By checking this box I authorize AIRC to forward to CINECA my contact data, the abstract of the application and the information included in this form (only if the grant application is funded).

An **early stage researcher** will be enrolled to conduct experiments planned in the research program.

I will post a job position for a junior postdoc with the following qualifications/skills:

Mandatory:
- Handling of transgenic mouse models and maintenance of colony;
- Previous experience with mouse cancer models, preferably melanoma;
- Administration of drugs by intraperitoneal injection and gavage;
- Mouse tissue sample collection;

Optional:
- Proficiency in the following molecular biology techniques: nucleic acid extraction, PCR, qrRT-PCR, western blotting, immunoprecipitation, chromatin immunoprecipitation and genotyping;
- Previous engagement in immunofluorescence staining to be evaluated by Confocal microscopy and Flow cytometry.

In case of missing optional skills, I will train personally the enrolled postdoctoral fellow to become proficient with the molecular and imaging techniques sharing all my knowhow. I do not envisage any criticism to supervise the early stage researcher thank to my previous experience with 3 PhD students and 2 Technicians.

These qualifications will allow to the enrolled post-doctoral fellow to perform most of in vivo experiments and to take care of mouse breeding to maintain transgenic mouse model colonies used in project proposal. The early stage researcher will perform genotyping, will subcutaneously inject syngeneic BRAF mutant mouse melanoma cells in 1) iEC^{tomato}; and 2) $Zeb1^{iEC-/-}$ mice, and will administer Anti-PD1; PLX4720; TGFβ/Zeb1 circuitry inhibitors. Then, he/she will collect melanoma samples and will take care of immunofluorescence and immunohistochemistry analyses to dissect the role of tumor endothelial cell in melanoma resistance. His/her skills in molecular biology will be exploited to perform PCR, qRT-PCR, western blotting, immunoprecipitation and chromatin immunoprecipitation.

DIPARTIMENTO
DI MEDICINA MOLECOLARE

SAPIENZA
UNIVERSITÀ DI ROMA

Rome, 21st June 2020

Letter of collaboration to Dr Chiara Cencioni

Dear Chiara,
I would like to thank you very much for presenting me as a collaborator for your MFAG project proposal entitled **"Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy"**.

I completely agree with the idea that investigating the properties of the TGFβ/Zeb1 circuitry regulatory network and of the ZEB1 transcription factor in particular, is of pivotal importance in enhancing our understanding of tumor associated endothelial cell function in the onset of melanoma resistance. The analysis of the role of endothelial cells on leukocytes trafficking, as depicted in your project plan, will be a major innovation in the field of endothelial dysfunction-driven melanoma resistance to chemotherapy and immunotherapy, since we currently have only partial and dis-homogenous information about.

My group has a long-standing interest in mechanisms harnessing leukocyte adhesion and transendothelial migration. We are actively investigating chemokine role in the regulation of leukocyte trafficking and how this affects diseases, i.e. multiple myeloma. Moreover, we are studying NK cell-based anti-tumor therapeutic approaches based on chemokine receptor targeting. In my group, all team members have expertise in molecular biology, cell biology, and immunology techniques, which could support experiments described in the research plan.

I am very happy to share resources and expertise with you and to collaborate on these subjects.
Please do not hesitate to contact me if you need further information.

Looking forward to starting this project,

Prof Giovanni Bernardini
Department of Molecular Medicine, University
of Rome "La Sapienza". V.le Regina Elena, 291. 00161- Rome, Italy
email: giovanni.bernardini@uniroma1.it

Università degli Studi di Roma "La Sapienza"
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DSTF
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UNIVERSITÀ DEGLI STUDI DI TORINO

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e-mail: direzione.farmaco@unito.it

Turin, June 15th, 2020

Subject: Letter of collaboration MY FIRST AIRC GRANT Call 2020

To whom it may concern,

I am writing this letter to confirm my support to My First AIRC grant application entitled: **'Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy'** presented by Chiara Cencioni, PhD as Principal Investigator.

My research group synthetized AA6 as a small molecule able to interfere with DNA methylation cycle. We are actively dissecting the exact mechanism of action of AA6. The collaboration with Dr Cencioni already revealed the anti-neoplastic potential of AA6 molecule in a mouse model of breast cancer-associated lung metastasis.
It will be very interesting to continue to collaborate with Dr Cencioni to extend the characterization of AA6 anti-neoplastic properties in melanoma. I am interested to study whether AA6 combination with current anti-melanoma drugs has an effect on therapeutic response and resistance prevention.

I am pleased to share with Dr Cencioni AA6 molecule and to provide any technical and scientific support required for the experimental activities planned in the present proposal.

I look forward for this project to start.

With best wishes,

Prof. Massimo Bertinaria (PhD)
Department of Drug Science and Technology
University of Turin
Via P. Giuria 9
10125 Torino - Italy
e-mail: massimo.bertinaria@unito.it
Phone: +39 011 6707146

DIPARTIMENTO DI ONCOLOGIA
UNIVERSITÀ DEGLI STUDI DI TORINO

Turin, June 16th 2020

Subject: AIRC My First AIRC Grant Call 2020

Letter of collaboration

To whom it may concern.

This letter is to confirm my willingness to continue the collaboration with Dr. Chiara Cencioni in the frame of the project submitted to AIRC "*Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy*".

My group has a long-standing interest in vascular biology and mechanisms influencing tumorigenesis and tumor progression. We are actively investigating the effect of novel combinatorial anti-tumor approaches aimed to enhance the therapeutic efficacy in melanoma mouse models of BRAF inhibitor and anti-PD1. I completely agree with the idea that investigating the properties of melanoma endothelial cells is of pivotal importance to clarify an overlooked vascular process, which promises being extremely important in chemotherapy and immunotherapy resistance onset. The molecular focus on TGFβ/Zeb1 circuitry will provide new therapeutic targets that will facilitate to overcome melanoma resistance.

I am already supporting this project that I find very novel and promising. I provided Dr Cencioni melanoma samples derived from mouse melanoma model upon treatment with PLX4720, anti-PD1 or both in combination. These samples allowed Dr Cencioni to point out an involvement of the transcription repressor factor Zeb1 in endothelial cells during response and relapse to PLX4720 treatment, which need to be further analyzed and characterized. I will continue to support Dr Cencioni studies providing all mouse melanoma samples she needs to dissect the role of melanoma associated endothelial cells in the onset of resistance to chemotherapy and immunotherapy. Members of my laboratory (Laboratory of vascular oncology) will continue to help Dr Cencioni in the *in vivo* experiments planned in her grant application to provide a molecular characterization of tumor endothelial cell contribution to melanoma chemotherapy and immunotherapy resistance and to highlight novel pharmacological targets promising prevention/reduction of melanoma endothelial anergy and mesenchymal transition. On vascular point of view I think to be very important to develop the concept of "endothelial anergy". Actually, in spite of the not terrific effect of anti-angiogenic regimens in the treatment of many solid tumors, the capillary vascular bed is necessary for tumor progression. Therefore, facing "endothelial anergy" represents a topic to expand the knowledge on the role of vasculature in tumor biology.

Università degli Studi di Torino - Dipartimento di Oncologia
Sede legale: c/o A.O.U. San Luigi Gonzaga - Regione Gonzole 10, 10043 Orbassano, To - Italy – Tel 0039 011 6705403–29, Fax 0039 011 6705413
Satellite Candiolo: c/o I.R.C.C. - Strada Provinciale142, km 3,95, 10060 Candiolo, To – Italy – Tel 0039 011 9933614, Fax 0039 0119933417
Satellite Torino: Via Michelangelo Buonarroti 27/bis, 10126 Torino - Italy - Tel 0039 011 6705306, Fax 0039 011 6705312

I am aware that my laboratory contribution will result in co-authorship in publications arising from the collaborative effort. The management of the resources will be carried out by Dr Cencioni, the Principal Investigator, who will assign to Laboratory of vascular oncology resources related to our role in the project.

Sincerely,

Prof. Federico Bussolino
Department of Oncology Director
University of Turin
Regione Gonzole, 10 – 10043, Orbassano (Torino)
e-mail: federico.bussolino@unito.it

Consiglio Nazionale delle Ricerche
Istituto di Biochimica e Biologia Cellulare
Institute of Biochemistry and Cell Biology

Monterotondo, June 19th 2020

Letter of collaboration to Dr Cencioni's MFAG application 2020

To whom it may concern

We hereby declare our intention and willingness to collaborate to the project entitled **'Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy'** whose Principal Investigator is Chiara Cencioni, PhD.

The CNR Monterotondo campus hosts an Animal facility, which operates in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) and supports researchers in their experimental activity. Within the facility, a specific Transgenic Animals Facility is available to generate genetically modified mice exploiting both standard (ESC blastocyst injection) and cutting-edge methodologies (i.e. CRISPR/Cas9 technology). The transgenic mouse facility is part of INFRAFRONTIER-Mouse Clinic Infrastructure and its management staff is represented by Dr F. Chiani and Dr A. Gambadoro. At moment the facility already produced about 140 mutant mouse lines and 50 mouse mutant CRE driver lines. The actual production capability of this team is about 15-20 mutant lines per years (http://www.ibcn.cnr.it/index.php/en/facilities/transgenic-facilities/monterotondo).

Within the project, we will share all our expertise and will generate an inducible endothelial Zeb1 knockout mouse (B6 -Zeb1$^{<tm1a(MFAG)Cnrm>}$ Tg(Cdh5-cre/ERT2)1Rha/Cnrm), which will allow Dr Cencioni to analyze whether the absence of Zeb1 expression in the endothelium will favor melanoma response to immunotherapy and will contribute to delay/prevent onset of immune checkpoint blockade resistance.
We will provide the inducible endothelial Zeb1 knockout mouse that will support the development of Dr Cencioni's grant proposal allowing the achievement of the related aims.

Yours faithfully,

Francesco Chiani, PhD and Alessia Gambadoro, PhD
CNR Institute of Biochemistry and Cell Biology
INFRAFRONTIER-EMMA-IMPC-Monterotondo Mouse Production-
32, Ramarini str. - 00015 Monterotondo (Rome, Italy)
Emails: francesco.chiani@cnr.it; alessia.gambadoro@cnr.it

ISTITUTO NAZIONALE TUMORI
REGINA ELENA
ISTITUTO DI RICOVERO E CURA A CARATTERE SCIENTIFICO

Rome, June 30th 2020

To Whom It May Concern:

I am writing this letter to confirm my support for Dr. Chiara Cencioni in her application for My First AIRC grant entitled: "**Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy**".

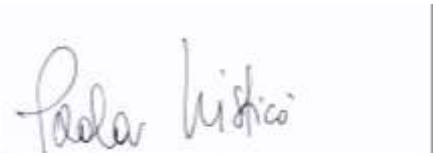
My lab is focused on understanding the immune response against tumor by studying the biological processes and signaling pathways involved in the complex interaction between tumor cells, extracellular matrix (ECM), cancer associated fibroblasts (CAFs) and immune cells. We have identified the actin cytoskeleton regulatory protein hMENA and its isoforms as crucial in ECM composition and immune cell localization in tumors with an impact on patients' prognosis. We work in close collaboration with our clinical department to identify TME-derived mechanisms of resistance to immune checkpoint inhibitor treatment and radiotherapy. Among the different pathways we are focusing on TGF-beta and its isoforms produced by stromal cells, such as CAFs. To this aim we are setting-up 3D models also bioprinted to recapitulate the interaction between tumor and stromal cells.

I believe that the research plan outlined by Dr. Cencioni is of great interest and that the analysis of the endothelial role during the development of melanoma resistance to immunotherapy can reveal novel attractive therapeutic strategies for the management of these cancer patients.

I am pleased to share with Dr. Cencioni all the knowhow of our group and to provide any technical and scientific support required for the experimental activities planned in the present proposal.

Please do not hesitate to contact me for any other information you may need.

Sincerely,



Paola Nisticò M.D.
Head of Tumor Immunology and Immunotherapy Unit
Department of Research, Advanced Diagnostic and Technological Innovation
Translational Research Functional Departmental Area
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Via Elio Chianesi 53
00144 Roma - Italy
Phone: +390652662539
FAX: +390652662600
e-mail: paola.nistico@ifo.gov.it

Gemelli

Rome, June 18, 2020

Re: MY FIRST AIRC GRANT Call 2020_ Collaboration letter

To AIRC,

I hereby declare my interest and willingness to collaborate in the project entitled **"Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy"** presented by Chiara Cencioni, PhD as Principal Investigator.

The Unit of Dermatology at the Fondazione Policlinico Gemelli, IRCCS, Rome is interested to understand the involvement of BRAF and other new loci in the genetic predisposition to both sporadic and familiar melanoma and to analyze clinical and molecular aspects of epithelial neoplasia. I strongly believe in the interaction between the clinic and the laboratory research.

It is exactly in this context that I support the study proposed by Dr Cencioni providing her clinical and histopathological information regarding melanoma patients stratified by disease stage and follow-up data. Her studies will allow to conduct a retrospective analysis to highlight tumor associated endothelium dysfunction possibly involved in endothelial anergy and dedifferentiation responsible of the onset of resistance to anti-neoplastic therapies. I will share my expertise on melanoma to support Dr Cencioni in the interpretation of her experimental data on human samples focusing on patients experiencing immunotherapy resistance.

I understand that I will be co-authors for any publication arising from these collaborative efforts, but do not expect to be entitled to intellectual property rights on the findings generated by this proposal.
I wish Dr. Cencioni every success with her grant application.

Yours Faithfully,

Prof. Ketty Peris

Full Professor at the Faculty of Medical and Surgery
Department of translational Medicine and Surgery
Catholic University of Sacred Heart – Fondazione Policlinico A. Gemelli IRCCS, Rome
e-mail: ketty.peris@unicatt.it

ISTITUTO DI CANDIOLO
IRCCS - Istituto di Ricovero e Cura a Carattere Scientifico

DIREZIONE SCIENTIFICA DI ISTITUTO
Direttore Professoressa Anna Sapino

Turin, June 25th, 2020

MY FIRST AIRC GRANT Call 2020_ Collaboration letter

To AIRC,

With this letter, we want to certify the collaboration with Dr Chiara Cencioni concerning the grant proposal entitled: **'Insight into TGFβ/Zeb1 circuitry promoting melanoma immunotherapy resistance through endothelial cell anergy'.**

The mission of our research conducted at the Surgical Pathology Unit of Candiolo Cancer Institute aims to understand the biological features of cancer lesions and to improve and develop novel technologies and biomarkers convenient for personalized therapeutic approach of cancer patients. Our research is focused mainly on melanomas, breast and colon cancers. The laboratory performs routinely different histological analyses exploiting the operative facilities for immunostaining, genetic and molecular analyses, producing medical reports for the management of cancer patients.

Understanding the contributive role of tumor associate endothelial cells in the onset of melanoma resistance to chemotherapy and immunotherapy represents a hot topic in the field of molecular oncology. We will provide Dr Cencioni melanoma sample slices derived from a cohort of 30-40 melanoma patients stratified by disease stage and follow-up data to conduct a retrospective analysis aimed to highlight tumor associated endothelium dysfunction possibly involved in endothelial anergy and dedifferentiation.

We will provide the help necessary for the development of this specific issue described in the project work plan.
We wish Dr. Cencioni every success with her grant application.

Warmest greetings,

Best regards

Prof. Anna Sapino
IRCCS-Candiolo Scientific Director

011.9933.465 direzione.scientifica@ircc.it