Immunoregulatory drivers of melanoma progression and resistance to therapy

Abstract.

CLINICAL NEEDS. Improvement of clinical management of cutaneous melanoma faces two apparently unrelated, but urgent needs: a) defining more effective prognostic factors in early disease, predicting the risk of clinical progression, compared to conventional histo-morphological factors; b) understanding which relevant genes control primary resistance to the most advanced treatments, as immunotherapy, in the metastatic setting. These two relevant goals are in fact strictly related, since common biological processes, under control of the same master genes, may contribute to foster clinical progression in primary disease and to induce therapy resistance in metastatic melanoma.

MAIN HYPOTHESIS. That clinical progression in primary disease and resistance to immunotherapy in advanced melanoma may be influenced by shared mechanisms, depending on action of master genes regulating melanoma phenotypic differentiation and interaction with the immune system. The latter processes are expected to shape the tumor immunoregulatory profile, thus contributing to immune suppression. We further hypothesize that: a) expression of differentiation-related and immunoregulatory genes will correlate with the immune contexture of primary and metastatic lesions; b) shared master genes can be identified that, when/if associated with an immunosuppressive tumor microenvironment, will behave as prognostic factors in early stage and as predictive factors of resistance to immunotherapy in advanced stage; c) mechanistic studies, based on gene silencing and pharmacological inhibition, will show that it is possible to revert the immunosuppressive gene expression profile of melanoma by targeting immunoregulatory and differentiation-related genes.

MAIN AIMS.

1. To develop improved prognostic algorithms in primary melanoma based on integrated analysis of differentiation-related markers and of the immune contexture.
2. To identify shared master immunoregulatory genes in primary and metastatic lesions, by correlating immune contexture analysis with transcriptomic profiling, and to test that such immunoregulatory genes have prognostic or a predictive roles.
3. To provide mechanistic insight by gene targeting for the role of genes investigated (aim1) or identified (aim2) in shaping the immunoregulatory profile of the tumor.

EXPERIMENTAL DESIGN AND METHODS. TASK1. We will use immunohistochemistry, multispectral digital pathology techniques and targeted NGS sequencing to test candidate markers encoded by genes regulating melanoma differentiation, markers defining different immune cells, and most frequent gene mutations, as new prognostic factors in primary melanoma. TASK2. By whole genome gene expression profiling coupled to immune contexture analysis we will identify genes significantly associated with an immunosuppressive tumor microenvironment and shared between primary and metastatic melanomas. TASK3. We will validate the prognostic/predictive value of genes and markers assessed in TASKS 1-2 in independent sets of primary and metastatic lesions. TASK 4. By gene targeting and pharmacological inhibition we will gain mechanistic insight on the immunoregulatory function of the genes investigated or identified in TASKS 1 and 2, respectively.
RELEVANCE OF THE EXPECTED RESULTS. 1. Definition of an improved prognostic algorithm in primary melanoma based on the integrated analysis for immune contexture and candidate immunoregulatory genes. 2. Demonstration that a set of differentiation- and EMT-related markers/genes are associated with bad prognosis in early stage melanoma and predict resistance to immunotherapy in late stage.

Background, state of the art, preliminary data.

1. The main clinical needs. Clinical management of cutaneous melanoma faces distinct but related needs depending on the stage of disease. In early, Stage I/II primary tumors, the most urgent hurdle to be overcome is the lack of more effective prognostic markers of progression and bad prognosis, since the current histo-morphological classification (AJCC 7th edition staging system) captures only a fraction of the molecular and biological heterogeneity of the primary lesions (1). In Stage III and IV, advanced disease, target therapy by BRAF and MEK inhibitors, and immunotherapy directed at immune checkpoints, have remarkably improved PFS and OS, however only a fraction of patients respond and both intrinsic and acquired resistance mechanisms need to be addressed, by promoting the identification of predictive factors of resistance to therapy (2). These different clinical needs in early vs late stage melanoma are in fact much less unrelated than it appears, as current evidence suggests that some common biological processes may contribute to clinical progression in primary disease as well as to target therapy and immunotherapy resistance in metastatic melanoma.

2. Why the current prognostic classification in early stage melanoma needs to include additional factors. The AJCC 7th edition staging system (1) identifies three main prognostic factors (thickness, ulceration and mitotic rate). Nevertheless, a great deal of uncertainty remains in the ability to predict 10-year survival rates for Stage I/II tumors. In the instance of thin (< 1mm) T1 melanomas, 10-year survival can range from 99% to 85%. However, some patients with <0.76 mm primary tumors can undergo progression to metastatic disease. On the other hand, there are patients with >4 mm primary lesions who never progress to stage III and remain alive without disease at >20 years after diagnosis.

3. Prognostic relevance in primary melanoma of the immune profile and of genes that control melanoma differentiation. The immune reaction in primary melanoma has a key prognostic role, as documented in recent years even in several additional solid tumors including colorectal, lung, breast and bladder carcinomas. Almost 30 years ago, W. Clark and colleagues showed that tumor-infiltrating lymphocytes (TIL) are independent prognostic factors in stage I melanoma (3). Subsequently, presence of TIL in the primary tumor has been shown to predict a negative sentinel node, suggesting an anti-metastatic effect of the immune response. Emerging evidence indicates that the immune contexture of the tumor (= type, density, position and functional aspects of immune cells that affects prognosis) can be shaped by, or it is frequently associated with specific transcriptomic profiles which in turn may have prognostic relevance. The TCGA network study, in >330 melanomas (20% primary lesions and 80% metastatic lesions) has identified a subset of tumors characterized by an immune-related gene signature, associated with better prognosis, increased burden of nonsynonymous mutations (which may generate neoantigens recognized by T cells) and enhanced infiltration by lymphocytes compared with tumors expressing the alternative "keratin" or the "MITF low" gene signatures. However, it is not clear whether the immune-related gene signature has similar prognostic role in both the advanced and the early tumors that may express such trait.
Murine models have been used to identify genes as ACPS that control tumor invasiveness and promote metastasis and, at the same time, are prognostic in primary human melanomas. In primary melanomas, a switch in favor of high expression of the EMT inducers ZEB1 and TWIST1, promoted by activation of oncogenic BRAF/NRAS, is associated with dedifferentiation (=suppression of the MITF program) and predicts shorter time of progression to metastatic disease (4).

4. Genes involved in regulating melanoma differentiation and interaction with the immune system contribute to resistance to therapy in advanced disease. As shown initially by our laboratory (5), advanced melanoma lesions can be classified in two large subsets defined by opposite expression of MITF and AXL and of related gene programs. Recently Tirosh et al. (Science 2016) have shown that melanomas can show an intra-tumor heterogeneous profiles with variable fractions of cells characterized by the "MITF program" (more differentiated cells) or by the "AXL program" (de-differentiated cells with a more invasive gene expression profile). Interestingly, increased frequency of AXL+ cells was associated with emergence of resistance to target therapy, with higher proportion of cancer associated fibroblasts (CAF), as well as with enhanced expression, in the tumor, of gene signatures defining immune cells (T, B and macrophages), compared to tumors characterized by a dominant MITF program. The AXL-/MITF+ signature has been independently correlated to intrinsic resistance to BRAF and MEK inhibitors. Moreover, enhanced expression of AXL, as well as of genes involved in the EMT process (such as WNT5A, TAGLN, LOXL, TWIST2) and in the recruitment of macrophages (CCL2, CCL7, CCL8, CCL13) defines tumors with intrinsic resistance to immunotherapy targeting the PD-1 immune checkpoint. Development of resistance to MAPK inhibitors in melanoma leads to upregulation of the YAP transcriptional co-activator and recent evidence indicates that this gene promotes recruitment at tumor site of suppressive myeloid-derived suppressor cells (MDSCs).

5. The need for developing an integrated analysis of the tumor immune contexture. The identification of new immune-related and robust prognostic and predictive markers is hampered by the complexity and heterogeneity of the tumor microenvironment. Within a heterogeneous neoplasm (where the process of branching evolution leads to an ever increasing molecular complexity) several different stromal and inflammatory cell types contribute to shape the biological behaviour of the tumor. Current approaches to deciphering the complexity and heterogeneity of the tumor microenvironment, and of the tumor-stroma cross-talk, are based on either the analysis of single cell suspensions from surgical samples (retaining ability for multi-marker quantitative analysis, but destroying the information on topological relationships within the tissue) or on semi-quantitative tissue characterization, "one marker at a time" with poor ability for multi-marker analysis (conventional immunohistochemistry). The latter approach lacks the ability to achieve a quantitative and comprehensive description of the tumor microenvironment. Such description needs to include information on cell types exerting anti- or pro-tumoral roles (T cells, regulatory T cells, macrophages, NK cells, MDSCs, just to name a few of the most relevant), on cell density, and on topological relationships among different cell types. The current project intends to overcome these limitations by developing the simultaneous, quantitative, multi-marker analysis of different immune cell types on a single tumor section through multispectral digital pathology analysis. This approach holds the promise for: a) generating an unprecedented level of quantitative and qualitative information on the immune contexture of neoplastic lesions that may be translated into prognostic and predictive algorithms; b) allowing to correlate the immune contexture with tumor morphology (=differentiation) and EMT-related programs (based on simultaneous staining for EMT-related tumor markers) in
primary and metastatic lesions; c) allowing, for the first time, to address intra-tumor heterogeneity in terms of correlation between the type of immune contexture features and specific differentiation or EMT-related traits in different tumor areas within the same lesion.

*Preliminary results.* The opposite proliferative/invasive melanoma programs exemplified by differential expression of MITF and AXL, and the relationship between melanoma de-differentiation and EMT programs have been investigated in advanced tumors. In agreement, the existence of melanoma subsets with opposite phenotype for MITF and AXL, as well as for expression of distinct EMT-related markers such as ZEB1, N-Cadherin, alpha-catulin and SNAIL (not expressed in E-cadherin+ tumors) was documented by western blot analysis on cell lines generated from surgical samples of metastatic melanoma patients (Figure 1). However, no information is available on the relationships of these markers/programs in thin primary lesions and on the potential association of a differentiated or EMT-like profile with the type of immune contexture observed in the lesions. To begin to address these issues we carried out a preliminary immunohistochemical analysis on panels of thin primary lesions (all previously characterized for conventional histopathological features including thickness, ulceration, mitoses, regression) by staining for CD3, CD8, HLA-Class I, HLA-DR, beta-catenin, COX-2, MITF, AXL, NGFR (CD271), S100, and PD-L1. Interestingly (Figure 2), we found presence of peri-tumoral (A) or intra-tumoral (B) CD3+ lymphocytes in MITF+ melanomas lacking AXL, and variable expression of PD-L1 on tumor or stromal components associated with frequent beta-catenin expression and variable positivity for COX-2. These initial results provide the rationale for integrating the analysis of the immune contexture with that of markers defining differentiation and EMT-like programs.

**References.**
Hypotheses.

1. That more effective prognostic algorithms, able to improve prediction of progressive disease in primary melanoma, compared to the current histo-morphological classification, can be developed by a comprehensive immunohistochemical and multispectral digital pathology analysis of lesions that integrates two sets of parameters: a): markers defining the immune contexture of the lesions, with emphasis on cell types that can exert and anti-(T) or a pro-tumoral (macrophages, myeloid lineage and Tregs) effect; b) candidate prognostic factors, expressed in tumor cells, and known to be involved in the regulation of melanoma differentiation and interaction with the immune system (such as AXL, MITF, ZEB1, TWIST1, COX-2, beta-catenin, PTEN, YAP, NFATc2, Myc, EZH2, FOXM1).

2. That immunohistochemical analysis of primary lesions for the markers defined in hypothesis 1 can identify: a) topological and statistically significant relationships between tumor markers associated with suppression of adaptive immunity (such as COX-2, beta-catenin, loss of PTEN) and deficient T cell infiltration/activation of the same lesions; b) topological and statistically significant relationships between expression of markers associated with recruitment of MDSCs (YAP) and actual presence of suppressive myeloid cells (CD163+, S100A9+ IDO+); c) association between tumor morphology (epithelioid vs. spindle) and expression of markers associated with de-differentiation (AXL, CD271) or EMT (ZEB1, TWIST1) and presence of a dominant immunosuppressive immune contexture; d) association between some of the candidate prognostic factors defined in hypothesis 1 and constitutive expression of PD-L1 and of other immune checkpoint ligands on melanoma cells with relationship with the type of immune contexture of the lesion.

3. That analysis for main genomic alterations (by targeted NGS sequencing of genes frequently altered in primary or metastatic melanoma) can associate some of the altered genes (namely those associated with increased nonsynonymous mutational burden such as NF1 and BRCA2) with specific immune contexture classes and the related gene signatures in primary and metastatic lesions.

4. That analysis of the transcriptomic profiles in primary and metastatic lesions can identify shared master genes that: a) are associated with immunosuppressive immune contextures; b) have prognostic significance in primary melanoma and predict resistance to immunotherapy in advanced disease; c) show differential expression in distinct areas of the same lesion in relationship with the local immune contexture, the tumor morphology and the expression of differentiation markers.

5. That targeting of candidate (defined in hypothesis 1) and new genes (defined in hypothesis 4) may reverse the immunosuppressive gene expression profile of the tumor.
AIMS.

1. To develop an improved prognostic algorithm in primary melanoma that may predict the risk of clinical progression by integrated analysis for the immune contexture of the lesions and for genes that regulate melanoma differentiation and epithelial-mesenchymal transition (EMT).

2. To identify new shared immunoregulatory genes in primary and metastatic melanoma associated with an immunosuppressive immune contexture and exerting a prognostic role in primary tumors and a predictive role of resistance to immunotherapy in advanced tumors.

3. To verify that targeting of genes mentioned in aims 1 and 2 can revert the immunosuppressive gene expression profile of the tumor.

Proposal main body

A. EXPERIMENTAL DESIGN.

TASK 1. Testing whether an integrated analysis of the immune contexture and of candidate differentiation-related markers allows to identify improved prognostic profiles in primary melanoma. 1.1. The analysis for presence, density, topological relationships, lineage and predicted pro- or anti-tumor function of cells belonging to the innate and adaptive arms of the immune system in the tumor microenvironment allows to define the so called “immune contexture” of a neoplastic lesion (1). By implementing immunohistochemistry and using multispectral digital pathology analysis we will characterize the immune contexture of primary lesions by looking at a limited but highly informative set of T cell, regulatory T cells and pro-tumoral macrophage markers including: CD3, CD4, CD8, PD-1, PD-L1, FOXP3, CD68, CD163, S100A9, IDO. By digital image analysis, immune cell density (cells/mm^2) and topological distribution (intra- vs. peri-tumoral) will be recorded. The characterization for the immune profile of the lesions will be coupled, and put into statistical correlation, with the analysis for expression of a set of candidate differentiation- and EMT-related markers. The main goal will be to test whether such integrated analysis allows to build a prognostic algorithm with improved ability to predict patients at risk of clinical progression, compared to conventional histo-morphological factors of the current AJCC Staging system (see methodology and statistical analysis section for statistical analysis related to data generated in this TASK). The set of candidate markers will include the products of genes involved in melanoma differentiation (MITF, AXL, NFATc2, Myc, Brn-2), EMT (ZEB1, TWIST1, EZH2, FOXM1) and in the regulation of the immune microenvironment (PTEN, YAP, COX-2, beta-catenin). To this end, we will take advantage of a large database available at Unit 2 that contains all clinical, histological and follow-up information on patients treated over the past 15 years for surgery of primary melanoma. This database is integrated with the corresponding tissue bank of FFPE lesions from all the patients. On the basis of criteria chosen with the help of a statistician that will collaborate to the project (Dr. Valter Torri, Mario Negri institute), in the discovery phase, we will select two panels of archival FFPE sections of primary melanoma lesions from patients with <1 and >4 mm with fully annotated clinical parameters and long term follow-up. The available clinical dataset, with associated archival FFPE lesions includes >3,000 thin (<1 mm) primary lesions and >1,100 thick (>4mm) lesions removed from patients admitted for surgery to our Institute from
2002 to 2013. The panel will be selected as to include, in each of the two thickness subsets, patients with short survival (<5 years) and long survival (>5 years). Within this TASK we will also address the issue of intra-tumor heterogeneity. To this end, we will use microdissection of areas showing different tumor morphology and presence/absence of immune cells to test the hypothesis that these different tumor areas are in fact associated with distinct expression of EMT- or differentiation-related or immunoregulatory genes as investigated by whole genome gene expression analysis (see methodology section).

1.2. Within this TASK we will test whether specific gene mutations in primary melanoma have a prognostic significance. Both neoplastic and normal tissues from each patient will be analyzed to this end. The characterization of genetic changes that accumulate during evolution of melanoma from precursor lesions has identified a limited set of key genes (as BRAF, NRAS, TERT, CDKN2A, PTEN and p53) (2). Some of these genetic changes may contribute to define prognostic subgroups and this may be true even for mutations affecting NF1 and BRCA-2 as described in the background section (3-5). Interestingly, the TCGA melanoma study included 20% of advanced primary tumors and several (3) NF1-mutant primary lesions were found. Our hypothesis is that targeted NGS sequencing for genes involved in melanoma genesis and progression, in a set of primary lesions that will include <1mm and >4mm thick lesions, may uncover subsets where specific gene mutations are associated with worse prognosis, even within the same “conventional” (=based on AJCC 7th edition) prognostic subset. The mutational profile will best statistically correlated to all conventional histopathological prognostic factors as well as to the immune contexture of the lesion, looking for significant associations (see methodology and statistical analysis section).

**TASK 2. Identification in primary and metastatic melanomas of shared immunoregulatory genes with prognostic and predictive value.** As a discovery set, we will select a panel of primary melanomas (with the same criteria as in TASK 1) as well as a panel of advanced or pre-therapy metastatic lesions from patients treated at our Institute with antibodies directed to immune checkpoints (anti-PD-1). Lesions will be initially characterized for the immune contexture and classified in a minimum set of 5 “immune related classes” according to the following criteria: class 1 “dominant anti-tumoral immunity”: lesions showing a marked infiltration by activated PD-1+ T cells, with low/absent presence of FOXP3+ lymphocytes and of potentially pro-tumoral myeloid cells (markers as in TASK 1); class 2 “mixed immunity”: lesions characterized by a mixed profile of infiltrating, activated T cells concurrently with marked presence of regulatory T cells and potentially pro-tumoral myeloid cells; class 3 “non functional/excluded immune response” presence of peri-tumoral T cells lacking expression of activation markers as PD-1; class 4 “dominant pro-tumoral immunity”: lesions characterized by low/absent presence of infiltrating T cells, but characterized by marked density of potentially pro-tumoral myeloid cells and regulatory FOXP3+ lymphocytes; class 5: “immunological ignorance”: lesions characterized by lack/paucity of immune cells (either T cells or myeloid cells). Within this TASK we will also address the issue of intra-tumor heterogeneity. To this end, we will use microdissection of areas showing different tumor morphology and presence/absence of immune cells to test the hypothesis that these different tumor areas are in fact associated with distinct gene programs. We will then carry out whole genome gene expression profiling of primary and pre-therapy metastatic lesions by the Ion Ampliseq Transcriptome Human Gene Expression technology. By BRB array tools software we will look for differentially expressed genes in
the 5 immune contexture classes. Upstream regulator analysis under the IPA software will then be used to identify master immunoregulatory genes significantly activated or suppressed in each of the 5 immune contexture classes. We will specifically look for genes whose level of expression is significantly associated with the same immune contexture class in both primary and metastatic lesions. The focus of the analysis will be on the genes overexpressed in immune contexture classes 2 to 5, while we expect that genes overexpressed in class 1 will mainly be those involved in positive regulation of adaptive immunity. We will test the hypothesis that the most promising candidates associated with negative regulation of immunity will be differentiation-related and/or EMT-related genes. These genes will be validated first by qPCR and then, at the protein level, by immunohistochemistry in the corresponding lesions. At the prognostic/predictive level the most promising candidates will be tested, by immunohistochemistry for the prognostic relevance in thin vs thick primary tumors, as well as in pre-therapy lesions from patients treated with immunotherapy targeting immune checkpoints (PD-1) and classified as being responders (clinical benefit) or progressors.

**TASK 3. Validation of the prognostic and predictive value of candidate markers (from TASK 1) and new immunoregulatory genes (from TASK 2) in independent panels of primary and metastatic melanomas.** The candidate markers (TASK1) and the new genes (TASK2) characterized in the previous TASKS will be validated for their prognostic or predictive value in the following independent sets of lesions: a) for the prognostic significance, the expression of the genes of interest (at the protein level by immunohistochemistry) will be evaluated in a set of matched primary/metastatic lesions from n=270 patients (all with fully annotated clinical records and long term follow-up). At the predictive level we will use: a) panels of pre-therapy lesions from (n=40) Stage IIIc or Stage IV resected melanoma patients treated with the association of anti-CTLA-4 and anti-PD-1 at our institution (all with complete clinical records) in the adjuvant setting. In this experimental set up we will test whether expression of the markers of interest characterize patients who progressed early on during adjuvant immunotherapy treatment. b) Panels of pre-therapy metastatic lesions from patients treated with anti-PD-1 antibodies. In this setting we will correlate the expression of the genes of interest with response (clinical benefit) or resistance (progressive disease). See Methodologies and Statistical Analyses for details on statistical analysis of data generated in this TASK.

**TASK 4. Mechanistic insight on immunoregulatory function of candidate markers (from TASK1) and new genes (from TASK2) found to have prognostic and predictive value.** As outlined in the background section, current evidence indicates that genes involved in the regulation of melanoma differentiation and the EMT process have a strong role in the resistance to therapy, including immunotherapy, in metastatic disease. In this task we will use gene silencing (either by siRNA or shRNA) and pharmacological inhibition to test the consequences of targeting the master genes described in TASK1 (AXL, MITF, ZEB1, TWIST1, COX-2, beta-catenin, PTEN, YAP, NFATc2, Myc, EZH2, FOXM1), as well as the new genes identified in TASK2, on melanoma differentiation, EMT profile and immunoregulatory gene expression. Effects of targeting these genes will be evaluated by comparison of cells with expressed vs silenced/inhibited genes for whole genome gene expression analysis. We will specifically look for the effects of gene targeting on expression of genes that may impact on
the inflammatory profile of the tumor (such as genes encoding chemokines, cytokines and growth factors, master genes of inflammation as those within the NFκB pathway, genes in the Type I IFN pathways and so on). To this end we will also use PCR, western blot and immunofluorescence analyses. The main goals of these experiments are: a) to get evidence that targeting of some of these genes can reverse the immunosuppressive gene expression profile of the tumor; b) to obtain evidence that some of these genes are “druggable”, i.e. that can be targeted by available inhibitors, thus providing a pre-clinical rationale for future combination therapies.

EXPECTED RESULTS

1. Definition of an improved prognostic algorithm in primary melanoma based on the integrated analysis for candidate differentiation- and EMT-related markers and for the immune contexture.
2. Demonstration that primary and metastatic melanomas can share a set of immunoregulatory genes that: a) correlate with specific immune contexture classes; b) are associated with bad prognosis in early stage melanoma; c) predict resistance to immunotherapy in late stage disease.
3. Identification of “druggable” immunoregulatory genes whose targeting by existing inhibitors may revert the immunosuppressive gene expression profile of the tumor.
METHODOLOGIES AND STATISTICAL ANALYSIS

Study design. Patients with completely resected, histologically confirmed primary (Stage I-II) and metastatic (Stage III-IV) melanoma will be eligible. Tissues will be used based on informed consent following approval of the project by the independent Ethics Committee of the Institute. The main strategy of the project, to maximize the chances of success in a relatively short time frame (<2 years to the first publishable results), is to focus the initial analysis for candidate prognostic factors (TASK 1) in primary melanoma on only two subsets of patients: those with T1 (<1 mm thickness) and those with T4 (>4 mm thickness) localized tumors. Within each of these subsets, two further groups of patients (=lesions) will be compared: patients with good survival (> 5 years) and patients with bad prognosis (survival <5 years). For investigation in metastatic melanoma, we will exploit advanced lesions as well as pre-therapy tissues from patients treated with anti-CTLA-4 or anti-PD-1 at our Institute over the past 6 years as well as during the time-frame of the project (n~100).

Sample size. The project will take advantage of the remarkable retrospective set of FFPE lesions from localized melanoma available at INT, with the associated fully annotated clinical records and long term follow-up information. The available clinical dataset, with associated archival FFPE lesions includes >3,000 thin (<1 mm) primary lesions and >1,100 thick (<4 mm) lesions removed from patients admitted for surgery to our Institute from 2002 to 2013. Moreover the Unit 2 database also includes a panel of ~270 patients for whom matched primary and metastatic FFPE lesions are available. This panel will be instrumental for validation studies. All mechanistic studies will be carried out by taking advantage of a collection of >100 melanoma cell lines isolated from advanced primary and metastatic melanomas in the laboratory of the PI and characterized for main driver mutations (6-10).

Characterization of the Immune contexture and of candidate differentiation-related markers. We will use both conventional immunohistochemistry as well as multispectral digital pathology based on the VECTRA3 (Perkin Elmer) instrument. Markers of choice will include those defining or associated with T, DC, macrophages, MDSCs and Tregs. The following markers will be assessed: CD3, CD4, CD8, PD-1, PD-L1, FOXP3, CD68, CD163, S100A9, IDO. Whole-section digital images stained by conventional immunohistochemistry will be acquired with the Aperio equipment and used to quantitatively assess the location and density of relevant immune cell subsets through Definiens Tissue Studio software. The VECTRA3 digital pathology equipment will be used to develop fluorescence-based, multi-marker assays, allowing to quantitatively determining the immune contexture and of the immunoscore (= the density and intra/peri-tumoral distribution of CD8+ T cells (1)) through simultaneous staining of single lesions with a combination of several antibodies for different immune-related or tumor-related markers. All non-immune markers will be investigated by immunohistochemistry and/or multispectral digital pathology.

Targeted NGS sequencing. The tumor genetic background of all the samples will be assessed through targeted next generation sequencing (NGS), that allows the simultaneous sequencing of a wide set of genes using a small amount of DNA extracted from formalin
fixed paraffin embedded specimens. In particular, we will design a specific panel targeting the main genes reported in the literature to be mutated in primary melanoma (2) and metastases (3). Genomic DNA will be extracted from formalin-fixed paraffin-embedded (FFPE) tumor and matched normal samples using the GeneRead DNA FFPE kit (Qiagen, Hilden, Germany). DNA amount and quality will be identified using Quibit following the manufacturer’s instructions. Targeted next generation sequencing (NGS) will be performed by using small genomic DNA (40ng), a Custom Panel (designed by ThermoFisher) targeting the coding sequences and promoters of selected genes frequently mutated in melanoma, the Ion AmpliSeq Library Kit2.0™ and the Ion Torrent SSXL™ platform (ThermoFisher) according to the manufacturer’s instructions. Sequencing data will be processed by using the Ion Torrent platform-specific software, Torrent Suite; the variant calling from sequencing data will be generated by using the Variant Caller plugin and variant calls will be, at first, annotated by Ensembl Variant Effect Predictor and Ion Reporter software and, then, manually examined by Integrative Genomics Viewer (IGV). Some mutations will be validated by Sanger sequencing.

**Gene expression profiling.** Independent biological replicates will be used from each lesion. For gene expression analysis we will use the AmpliSeq™ Transcriptome Human Gene Expression Kit, including the Ion AmpliSeq™ Human Gene Expression Core Panel, to prepare libraries from RNA, following standard procedures. This panel provides gene-level expression information from a single multiplexed panel targeting over 20,000 genes which cover >95% of the RefSeq gene database. The small amplicon designs enable the use of the panel with RNA isolated from FFPE tissues and requires 10 ng of total RNA input. No polyA selection or ribosomal RNA depletion is necessary for use. The panel is optimized to work with the Ion AmpliSeq™ Library Kit Plus and the Ion Xpress™ Barcode Adapters, enabling preparation of barcoded libraries that can be multiplexed, templated using Ion Chef™ or Ion OneTouch™ Systems, and sequenced using the ION S5 XL System. The analysis will be performed by the Ion Reporter software. All gene expression analyses will be carried out with replicate biological samples, to allow appropriate statistical analysis of differentially expressed genes. Differentially expressed genes among pre-defined melanoma classes will be identified by BRB array tools software. Master immunoregulatory genes will be identified by upstream regulator analysis tool under the Ingenuity Pathway Analysis (IPA) software as described (5,8,10). Genes of interest will be validated by qPCR and immunohistochemistry.

**Gene targeting.** Targeting of genes of interest will be carried out as described (7) by either siRNA or shRNA as well as by pharmacological inhibition with specific inhibitors. Whole genome gene expression analysis in cells bearing expressed or silenced genes of interest will be carried out on the Illumina platform of the Institute. Analysis of differentially expressed genes in control and gene-targeted cells will be carried out as described in the previous section, as well as by western blot and by ELISA or intracellular flow cytometry (for soluble molecules).

**Statistical analysis.** Data analysis related to TASK1 and TASK3. The prognostic value of three groups of biomarkers (BM) involved in 1) melanoma differentiation and EMT, 2) features of the immune contexture and 3) representing gene mutations present in primary melanoma will be evaluated. Outcome measure will be the progression free survival (PFS),
measured from diagnosis to occurrence of the event of interest (progression or death, whichever come first) or to the date of last observation available for patients alive without progression. Also the impact on overall survival (OS) will be analyzed. For each of the three groups the BMs will be tested for their association with outcome by means of a multivariate proportional hazard regression model. Using a backward approach the most parsimonious model will be selected; cut off p value for staying in the model will be \( \leq 0.05 \), two sides. Once that the three models will be derived, the BMs remained in the models, will be analyzed together in a further model, using the same selection modalities. The final model will be then analyzed for evaluating its capability of discriminating, by means of evaluation of Harrel’s test. Improvement of the information provided by this model vs. the prognostic value of classical clinical and histo-morphological factors will be analyzed, including in the final model also the clinical variables and evaluating the improvement by means of a roc analysis for survival data. Given the availability of a large sample of patients/lesions (see characteristics of the database mentioned in the sample size paragraph), the approach is able to guarantee a solid goodness of fit of the model.

Data analysis related to TASK2. Once the association between selected genes and the five types of immune contexture will be identified by means of a cluster analysis, their association with response will be tested by means of a multivariate logistic regression model; roc analysis will be used for evaluating their discriminant values in each of the relevant subgroups (thin vs thick primary tumors as well as in pre-therapy lesions from patients treated with immunotherapy targeting immune checkpoints.

**PITFALLS AND CAVEATS**

- We are aware that thin primary melanomas may have limited neoplastic tissue available for all the analyses planned in the project. However, the preliminary figures enclosed in the proposal show that it is possible to accurately evaluate a large set of marker even in these small lesions (tumors in the 1mm thickness range). Moreover, current targeted NGS sequencing and gene expression techniques rely on the usage of extremely limited amount of tissue. As an example, we verified (data not shown) that effective whole genome gene expression analysis can be achieved starting with 20 micron thick sections/biological replicate. Moreover, the Institute tissue bank maintains also frozen replicates of the FFPE lesions, thus providing a further source of tissue.
- We are aware that specific miRNA are involved in regulating the EMT process in melanoma. We will address the potential role of selected miRNA by qPCR.
- Performance of the Ampliseq™ Transcriptome Human Gene Expression Kit will be compared in selected instances with the RNA-Seq Illumina platform available at our Institute.
- Some patients undergoing immunotherapy with immune checkpoint blockers, may have previously received a different type of treatment (most frequently target therapy). The potential effect of previous therapy on the immune profile of the neoplastic lesions will be taken into consideration.
- The numerical size of panel of metastatic patients treated with immunotherapy at our Institute (around 100) may be a limiting factor for the validation studies. Should this need emerge, we will activate collaborative efforts with additional Cancer Institutes in Italy to expand such casistic.
References.
DESCRIPTION OF THE INTERDISCIPLINARITY AND SYNERGY OF THE PARTICIPATING UNITS (SPECIFY CONTRIBUTION OF EACH UNIT).

The development of the proposed project requires the availability of high quality biological samples, with the associated full clinical parameter annotation, as well as the integration of several different types of expertise, which are available in the team of units, and that will be integrated in a synergistic fashion. This approach will contribute significantly also to improve the professional competence of each unit and will be reflected in the expected output of papers. Furthermore, the in depth statistical analysis of all the data generated in the project will take advantage of the specific expertise of a statistician (Dr. Valter Torri, Mario Negri Institute, Milan). In detail, the project is structured as a collaborative effort that will integrate the clinical expertise of the Melanoma and Sarcoma Unit in the surgical treatment of primary and metastatic melanoma, of the Division of medical Oncology in the immunotherapy of advanced melanoma, and of the Division of Pathology in the morphological, histo-pathological and immune-related classification of primary and advanced melanomas, with the translational research experience of the Human Tumors Immunobiology Unit and with the experience of the Laboratory of Molecular Pathology in assessing the mutational profile of melanoma by targeted NGS sequencing and the gene expression profiles by Ion Ampliseq human transcriptome/ION S5 XL System. The Unit of the PI will: a) develop the immunohistochemical and multispectral digital pathology characterization of the lesions in close collaboration with the Pathology Unit; b) carry out all the in-vitro mechanistic studies to decipher the immunoregulatory mechanisms of selected differentiation and EMT-related genes; c) analyze gene expression datasets to identify master genes and signatures that correlate with immune contexture classes; the Melanoma and Sarcoma unit will take care of selecting all primary lesions to be investigated in the project and to integrate all biomarker data generated in the study with the existing clinical parameters; The Medical Oncology 1 unit will be in charge for the clinical management of advanced melanoma patients treated with immunotherapy and for the integration of clinical parameters with the biomarker data generated by the PI and by the Pathology Unit; the Pathology Unit will carry out all the assessments for expression in primary and metastatic lesions of differentiation- and EMT-related markers, as well as the immune contexture analysis of the lesions. the Laboratory of Molecular Pathology will carry out targeted NGS analysis of altered genes in melanoma lesions as well as analysis of the gene expression profiles of the FFPE primary and metastatic lesions.
SIGNIFICANCE AND INNOVATION.

**Significance:** a) providing a clinically applicable improved prognostic algorithm that may predict the risk of progression in primary melanoma. Such achievement has a practical impact on melanoma management: the early identification of patients at high risk of clinical progression will assist clinicians in planning appropriate adjuvant treatments and/or more frequent patients' re-evaluations. b) Identification of predictive factors of resistance to immunotherapy related to the melanoma differentiation, EMT pathways as well to immune regulation and shaping of the immune contexture. c) Identification of new potential therapeutic targets for reversing the immunosuppressive profile of melanoma lesions. d) Based on the relevance of the goals, and of the clinical needs addressed, the current proposal has the potential for representing a starting point even for future large national or trans-national collaborative studies within the framework of research programs of the EU.

**Innovation:** Implementation of multispectral digital pathology will allow for the first time to develop the integrated analysis of the immune contexture of melanoma lesions. Moreover, validation of the prognostic and predictive value of data generated by multispectral digital pathology has transformative potential for the immunopathology field in any solid tumor where information regarding the immune profile of the lesions is critical for therapeutic decision involving immunotherapy. To the best of our knowledge, at time this proposal is being written, multispectral digital pathology analysis of tissues has never been implemented in our country and it's just beginning to be used in high profile scientific journals in the context of immunotherapy studies.

GENERAL TRANSFERABILITY, POTENTIAL IMPACT OF RESULTS AND OUTPUTS (1000 words)

- The results of this project have the potential for changing the clinical management of early melanoma, by allowing the prompt identification of patients with localized melanoma, but at high risk of progression. Results will also be shared through presentation at national and international meetings.
- The identification of new predictive biomarkers of resistance to immunotherapy in metastatic melanoma has the potential for leading to more cost-effective use of existing antibodies targeting immune checkpoints, as such type of immunotherapy has a strong economic footprint on the National Health Service (NHS). Understanding which markers may predict non-responsiveness to immunotherapy may help clinicians in selecting the appropriate therapy for different patients, according to the molecular/immunological features of their tumor.
- Identification of new predictive markers of resistance to immunotherapy in advanced disease may also define new therapeutic targets, i.e. genes that when targeted by specific inhibitors may lead to a reversal of the immunosuppressive profile of a tumor. These results thus may open the way to development of new combination therapies.
- The results of the project will be shared with the international scientific community through publication on peer reviewed journals. Based on the strength of the collaborative group, it is expected that several high quality publications will be produced during the time frame of the project.