**ALLEGATO B**

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

## TRANSLATIONAL RESEARCH

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

## 1. Principal investigator's full name and qualification:

(Please include: CV in European format with list of publications; IF end Hi-index )

2. Proposal title **Study of AMBRA1 in spitzoid tumors: identification of a biomarker panel for improved diagnosis**

3. Primary area of Relevance **Translational Medicine**

4. Relevance for the National Health System **Secondary and Tertiary preventions**

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7. Proponent's signature **Valentina Cianfanelli**

8. Authorized Administrative Official's signature **Prof. Bruno Dallapiccola**

Il Direttore Scientifico
(Prof. Bruno Dallapiccola)

9. Place and date **Rome, 15/01/2021**

## SELF EVALUATION FORM

1. Investigator's full name: (PI) **Valentina Cianfanelli**
2. Total papers **29** IF **216,55**
3. Total papers (last 10 years) **29** IF **216,55**
4. Total Papers as first/last author or corresponding author **10**
5. Total H-index **15** (Source: Scopus); **16** (Source: Google Scholar)

## PROPOSAL MAIN BODY

1. **PROPOSAL TITLE** **Study of AMBRA1 in spitzoid tumors: identification of a biomarker panel for improved diagnosis**

2. **ABSTRACT**

Spitzoid neoplasms are a group of melanocytic tumors, also described as "melanomas of childhood", which include benign tumours (Spitz naevi), malignant tumours (spitzoid melanomas) and tumours with borderline histopathological features and uncertain clinical outcome (atypical Spitz tumours). The conflicting histomorphology of the atypical Spitz tumours poses a diagnostic challenge, which results in either under-diagnosis as benign Spitz naevi or over-diagnosis as spitzoid melanomas. Furthermore, our understanding of the genetic underpinnings of spitzoid neoplasms is extremely poor. Hence, additional studies aiming to better understand pathogenesis and biological features of spitzoid neoplasms and to identify novel diagnostic biomarkers are needed.
AMBRA1 is a scaffold protein regulating autophagy and cell proliferation. We recently characterized AMBRA1 as a tumor suppressor gene and our unpublished data shed new light on the relevance of AMBRA1 in spitzoid neoplasms' biology and etiology. In particular, we exploited a mouse model expressing pro-oncogenic determinants of the spitzoid neoplasms in the skin, which shows worse histopathological features upon Ambra1 depletion. Cyclin D1, p21 and p27 are cell cycle regulators commonly used in histopathology for the diagnosis of melanocytic lesions. However, their reliability in differentiating between atypical spitzoid tumors and spitzoid melanomas is extremely poor, due to unclear and limited data. Our data place cyclin D1, p21 and p27 as novel AMBRA1 targets. Altogether, this evidence candidates AMBRA1 as a novel diagnostic biomarker in spitzoid neoplasms.
In the light of our preliminary results, by this proposal we aim at: i) validating AMBRA1 as a novel diagnostic marker of spitzoid neoplasms, in combination with AMBRA1 targets also altered in this context (cyclin D1, p21, p27), ii) functionally validating the tumor suppressor role of AMBRA1 in melanocytes, and iii) testing the relevance of AMBRA1 targets in the AMBRA1-mediated tumor suppression. To address our aims, we will check for the correlation among AMBRA1 and AMBRA1 target levels in spitzoid tumors ex-vivo, on a significant cohort of patient samples (aim i). In addition, we will rely on a spitzoid melanocyte model, engineered for AMBRA1 depletion (aim ii), or expression of AMBRA1-mutant specifically impacting the levels of cyclin D1 (aim iii).
This approach will provide molecular insights and mechanistic information, which are critical for improving the diagnostic accuracy of spitzoid neoplasms at morphological and immunophenotypical level. Furthermore, our study will contribute to a better understanding of the pathogenesis of spitzoid neoplasms, hence adding to the knowledge necessary for the development of new targeted therapies for melanoma, with and without spitzoid

features.

## 3. INTRODUCTION

**Spitzoid neoplasms** represent uncommon melanocytic lesions and account for only about 1% of resected melanocytic neoplasms (Requena et al., 2009; Weedon and Little, 1977). Because of their predominant occurrence in children and adolescents, these tumors were first described as “melanomas of childhood” (Spitz, 1948). However, they can also occur later in life.

Spitzoid neoplasms are composed of large epithelioid and/or spindled-shaped melanocytes that show large nuclei with vesicular chromatin and prominent nucleoli. These lesions include both benign tumours (Spitz naevi) and malignant tumours (spitzoid melanomas), with the latter often showing aggressive clinical behaviour with widespread metastasis, similar to conventional melanomas. Tumours with borderline histopathological features and uncertain clinical outcome also classify as spitzoid neoplasms and are named atypical Spitz tumours. Atypical Spitz tumours are characterized by histological features overlapping those of benign Spitz naevi and spitzoid melanoma. Their capacity to metastasize is usually limited to the regional lymph nodes, and has little effect on patient survival (Ludgate et al., 2009).

Unfortunately, in the case of **atypical Spitz tumours**, diagnosis is very difficult and a high diagnostic variability is reported also among experienced pathologists. Morphology, in fact, does not provide definitive criteria for classification of these tumors and invariability fails to predict the biological potential and thereby the clinical outcome.

**AMBRA1** is a scaffold protein with a rich interaction network (Cianfanelli et al., 2015a). As a result, AMBRA1 is involved in several cellular pathways (namely autophagy, cell metabolism, cell death and cell proliferation) and crucially regulates cellular homeostasis and cell response to stress (*Cianfanelli* et al., 2015b).

Recently, we attributed to AMBRA1 a pleiotropic and fine-tuning activity in **targeting specific proteins for proteasomal degradation** [c-MYC (*Cianfanelli* et al., 2015c), FOXO3A (Becher et al., 2018), and ULK1 (Nazio et al., 2013)]. Accordingly, Ambra1 role is highly context- and cell type-dependent.

In the case of the cell cycle regulator and proto-oncogene c-MYC, AMBRA1 favors the interaction with its phosphatase PP2A, thus facilitating c-MYC dephosphorylation and degradation *via* the proteasome (*Cianfanelli* et al., 2015c). Similarly, our most recent and unpublished data report a novel **role for AMBRA1 in regulating D-type Cyclins, p21 and p27** abundance, by enhancing their proteasomal degradation (see Preliminary Results below). Furthermore, we show that AMBRA1-mediated regulation of c-MYC and D-type Cyclins impacts the cell cycle and counteracts tumor growth in several cancer models (*Cianfanelli* et al., 2015c). These findings corroborate the establishment of Ambra1 as a multifaceted tumor-suppressor.

In the light of its effect on both pro- (c-MYC, D-type Cyclins) and anti-proliferative (p21, p27) factors, AMBRA1 may have a context-dependent activity in cell proliferation, promoting opposite effects in different patho/physiological conditions.

## 4. BACKGROUND AND RATIONALE

Because of their morphology being poorly informative in terms of clinical outcome, *spitzoid tumors constitute a challenging set of lesions to diagnose*. This difficulty leads to under-diagnosis as naevus, to over-diagnosis as melanoma, or to a diagnosis of ‘melanocytic tumour of uncertain malignant potential’ (Barnhill et al., 1999).

In this regard, the genetic alterations frequently observed in spitzoid tumors are not helpful neither. Indeed, they often occur in all spitzoid tumors (both benign and malignant ones) and sometimes even in common melanocytic naevi (e.g. B-Raf$^{V600E}$). Such alterations

encompass for instance Tyrosine kinase fusions (Wiesner et al., 2014) and chromosomal aberrations (6q23, 9p21, 11q13).

The unclear etiology and pathogenesis of spitzoid neoplasms have led to numerous studies aiming to increase our knowledge on the subject (Tetzlaff et al., 2017). Along the same lines, because of the diagnostic difficulties, great effort has been put in identifying novel diagnostic markers.

However, *to date no set of biomarkers can predict whether an atypical Spitz tumor will behave benign or malignant in the future*. Therefore, there is growing interest in the identification of molecules which impact the outcome of these lesions, to be exploited as novel diagnostic biomarkers as well as therapeutic targets.

5. **PRELIMINARY RESULTS** (*Cianfanelli* et al., 2015c); Maiani*, *Milletti** et al., Nature 2021, in press; *co-first authors. *Cianfanelli* and *Milletti* are the PI and a team member for this proposal, respectively)

We recently identified **AMBRA1** as a **tumor suppressor gene** and investigated its impact on tumor insurgence, growth and aggressiveness of breast and lung cancer models (*Cianfanelli* et al., 2015c). In addition, we further characterized the mechanistic insights into AMBRA1-mediated regulation of cell proliferation, and discovered that **AMBRA1 deficiency results into accumulation of key cell cycle regulators**: namely **cyclin D1, p21 and p27** (Figure 1, our unpublished data: Maiani*, *Milletti** et al., Nature 2021, in press). In particular, AMBRA1 is necessary for the proteasomal-mediated degradation of the aforementioned cell cycle regulators, due to its direct interaction with specific E3 ligase complexes. Of note, we generated a mutant of AMBRA1 (AMBRA1 ΔWD40) uncapable of targeting cyclin D1 for proteasomal degradation, but still able to regulate other AMBRA1 targets (data not shown; Maiani*, *Milletti** et al., Nature 2021, in press).
Interestingly, **cyclin D1, p21 and p27 levels are high in spitzoid neoplasms**. However, we were prompted by the relative lack of literature about the diagnostic power of such data (Ewanowich et al., 2001; Kapur et al., 2005; Nagasaka et al., 1999; Stefanaki et al., 2007) to perform cyclin D1, p21 and p27 IHCs on a cohort of patients affected by Spitz tumours (Figure 2, our unpublished data). Interestingly, Cyclin D1 IHC in an atypical Spitz tumor of an adolescent shows a homogeneous nuclear expression in epithelioid melanocytic cells spanning from the dermal-epidermal junction to reticular dermis (full thickness). This feature is generally observed in malignant melanomas and not in benign melanocytic lesions (Figure 2a-c). Regardless, this spitzoid tumor never became malignant in a 10-year follow-up, thus showing that cyclin D1 de-regulation is not a reliable marker of cell cycle proliferation/malignant phenotype in this context. Along the same line, malignant cells are positive for both cyclin D1 (positive regulator of cell proliferation) and p21/p27 (inhibitors of cell proliferation) IHCs, once again suggesting that their levels are not predictive of the cell proliferation rate (Figure 2). Also, a preliminary morphological and immunophenotypical analysis has been performed in about 50 cases of Spitzoid lesions *ex-vivo* confirming the unexpected expression of Cyclin-D1 and other cell-cycle regulators in lesions with epithelioid morphology.

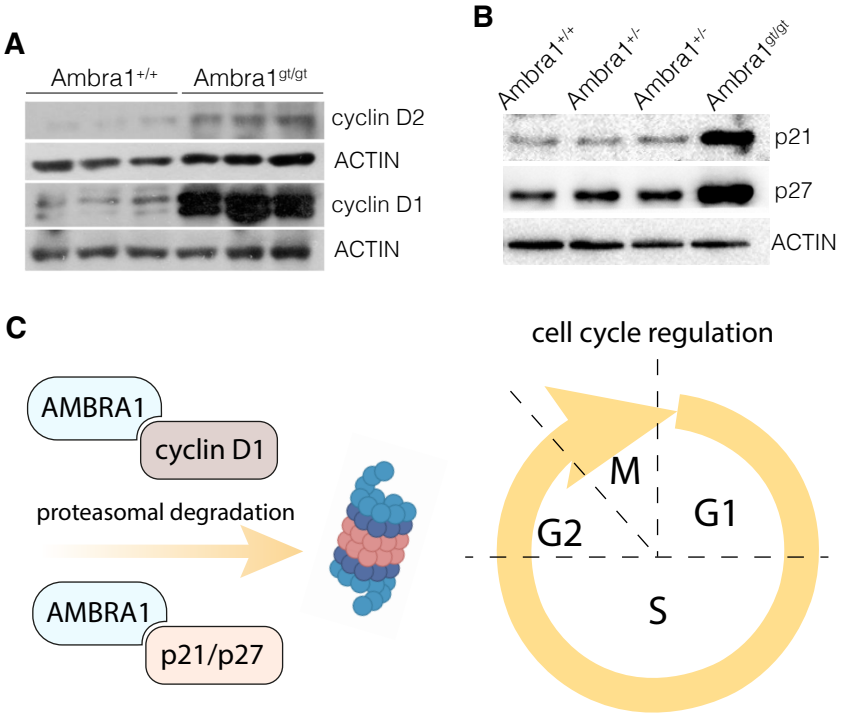


**Figure 1. AMBRA1 regulates D-type Cyclin, p21 and p27 proteasomal degradation.** **A-B**. Immunoblot on protein extracts from wild-type (Ambra1$^{+/+}$) and Ambra1 defective (Ambra1$^{-/-}$) embryos, isolated at embryonic day 12.5-13.5. **A.** Immunoblot of D-type Cyclins **B.** Immunoblot of p21 and p27 **C.** Model of Ambra1-mediated proteasomal degradation of D-type Cyclins and p21/p27.

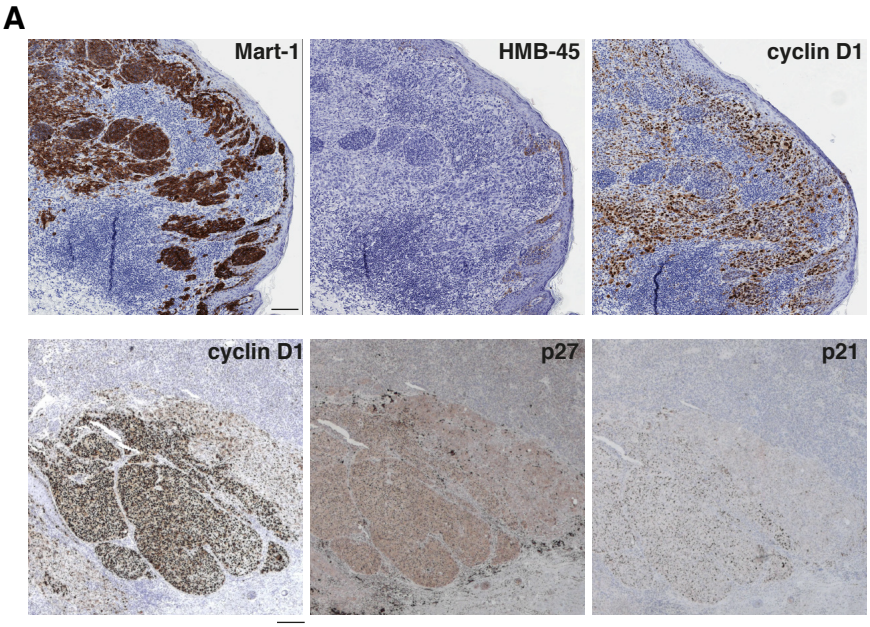


**Figure 2. Immunohistopathological analysis of spitzoid tumors**. **UPPER panels**, Immunohistochemistry labeling of the indicated proteins in a spitzoid melanocytic lesion, with mature melanocytic aggregates and epithelioid cellular elements. Melanocytes are stained with the lineage specific marker MART-1, while HMB-45 staining indicates the complete maturation of the lesion. At variance with the good prognosis of such nevus, staining for cyclin D1 shows a strong positive nuclear signal along the entire lesion. **LOWER panels**, Immunohistochemistry labeling in spitzoid melanocytic lesion for cyclin D1, p27 and p21, whose expression is limited to the malignant melanocytes.

This evidence raises the question of what **regulative mechanism(s) are responsible for the conflicting de-regulation of cell cycle proteins in spitzoid tumors**. Of note, their increased levels often do not correlate with gene amplification (Ewanowich et al., 2001; Kapur et al., 2005), thus suggesting a post-translation de-regulation. In the light of the functional link we found among AMBRA1 and cyclin D1, p21 and p27, we hypothesize here a relevance for AMBRA1 in spitzoid tumors. Thus, we performed a proof-of-concept experiment by exploiting a mouse model resembling spitzoid naevi/tumors, and selectively lacking Ambra1 in the skin (Figure 3, our unpublished data). Interestingly, **Ambra1 depletion worsen the histopathological features of the spitzoid mouse model**, resulting into hyperpigmentation of the hypodermal layer of the skin (Figure 3a-b) and hyperproliferation (Figure 3c-d).

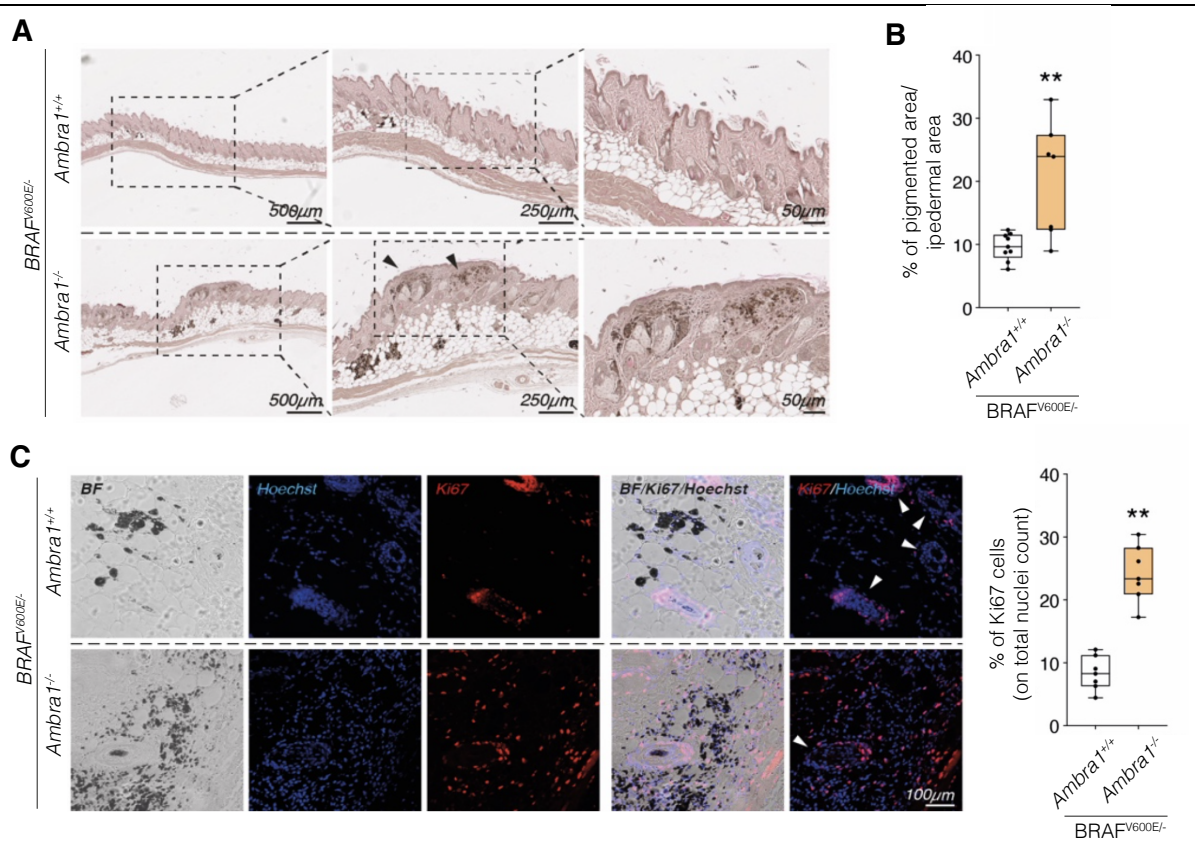


**Figure 3. AMBRA1 affect cells proliferation in an *in vivo* model of spitzoid neoplasm.**
**A**. We established a mouse model bearing a gene encoding for the constitutive active Tyrosine kinase B-Raf (B-Raf$^{V600E}$) and engineered for knocking-out Ambra1 gene (Ambra1$^{-/-}$). Both B-Raf$^{V600E}$ and Ambra1 depletion are under the control of CreER$^{T2}$, which allows their expression selectively in melanocytes, upon 4-OHT administration on the mouse skin. We administered 4-OHT to the dorsal skin of postnatal days 1, 3 and 5 *CreER$^{T2/+}$;Braf$^{V600E}$;Ambra1$^{+/+}$* and *CreER$^{T2/+}$;Braf$^{V600E}$;Ambra1$^{-/-}$* pups. Constitutive activated Braf, common in spitzoid tumors, when combined with *Ambra1* deletion displayed a significant increase in pigmentation of melanocytic lesions and increased proliferation (higher magnification). Areas of increased pigmentation were microscopically detected upon Hematoxylin and Eosin (H&E) staining of formalin-fixed and formalin-fixed paraffin-embedded (FFPE) skin sections, 13 weeks after 4-OHT administration. **B**. Quantification of the pigmentation of the hypodermal layer of the skin. **C**. LEFT, Cell proliferation was assessed by Ki67 immunostaining (red) in FFPE-skin sections of *Braf$^{V600E}$;Ambra1$^{+/+}$* and *Braf$^{V600E}$;Ambra1$^{-/-}$* RIGHT, Quantification of the Ki67 signal per nuclei.

## 6. AIM OF THE STUDY

Due to the lack of reliable biomarkers, the diagnosis of spitzoid tumors constitute a challenge, and often results into misdiagnosis. Also, the molecular mechanism(s) underlying the etiology of spitzoid tumors are poorly characterised. Interestingly, unlike conventional malignant melanomas, spitzoid tumors show a dissociation between the levels of cell cycle regulators and cell proliferation rate. This evidence calls for the identification of novel diagnostic markers and a better understanding of spitzoid tumor biology.

Based on these premises, **this pilot research project aims at disclosing the relevance of AMBRA1 in the diagnosis and etiology of spitzoid tumors**. In particular, we will address the following aims:

1. To assess AMBRA1 reliability as a putative diagnostic marker of spitzoid tumors (Task 1)
2. To evaluate the tumour suppressor role of AMBRA1 in a model of spitzoid naevi/tumors (Task 2)
3. To characterise the interplay among AMBRA1, cyclin D1, p21 and p27 in spitzoid tumor biology (Task 3)

## 7. EXPERIMENTAL DESIGN

**Task 1. Validation of AMBRA1 as a putative novel diagnostic marker of spitzoid tumors**

*Rationale:* Immunohistochemical approach is an essential application in surgical-pathology to properly classify neoplasms as a function of their prognosis. Cell-cycle regulator levels are conventionally considered reliable markers to infer the proliferative status of a neoplastic lesion.
However, in spitzoid lesions, the reliability of this approach is often compromised by the dissociation between the low proliferative status assessed by nuclear Ki-67 levels, and the high levels of cyclin D1, p21 and p27, possibly suggesting an aberrant regulatory network that includes these proteins without affecting malignant transformation. Since we identified AMBRA1 as a post-translational regulator of these cell cycle proteins (Maiani*, *Milletti** et al., Nature 2021, in press. See also Preliminary results and Figure 1), we will evaluate by means of different approaches AMBRA1 status in spitzoid tumors.
*Experimental Plan:* We will take advantage of an already established cohort of histological samples of spitzoid tumors (50 patients, mostly children/adolescents and young adults. Ethical protocol: CE n. 8391/2013 Roma; S. Andrea Ethical Committee) to comprehensively evaluate AMBRA1 levels in combination with cyclin D1, p21 and p27 through IHC. Of note, the cohort has been previously characterized for chromosomal aberrations so to be aware of any aberration on the *loci* of our interest (namely cyclin D1, p21 and p27).
Of note, information relative to the clinical outcome of our cohort of patients will be made available, and will allow us to correlate clinical outcome with the levels of markers of interest (AMBRA1, cyclin D1, p21, p27).
*Methods:* Tissue specimens from benign and malignant melanocytic lesions and Spitzoid tumors will be analysed by IHC, by using already tested antibodies for the recognition of human AMBRA1, cyclin D1, p21 and p27. Normal tissues surrounding the lesion will be used as an internal negative control for cyclin D1, p21 and p27. Also, markers of melanocytic lineage (MART-1, HMB-45), proliferation (Ki-67), and eventually stromal markers of malignant melanocytic transformation (Tenascin, Fibronectin E-B) will be considered to implement and corroborate our findings. Positivity score for immunophenotypical analysis will be defined by at least two independent pathologists.
Data will be finally analyzed, compared to clinical data and statistically evaluated.

**Task 2. Functional validation of the tumour suppressor role of AMBRA1 in spitzoid tumors**

*Rationale:* We already characterised AMBRA1 as a tumor suppressor gene in breast and lung cancer (*Cianfanelli* et al., 2015c). In addition, our preliminary data show that the lack of AMBRA1 increases cell proliferation also in a model of spitzoid naevi/tumors (Figure 3, unpublished data). Prompted by this evidence, we hypothesize that AMBRA1 plays as tumor suppressor gene in spitzoid tumors.
*Experimental Plan:* We will first exploit non-tumorigenic mouse melanocytes (Melan-a cells, (Bennett et al., 1987), already available in our laboratory. We will establish different Melan-a cell lines bearing genetic alterations commonly observed in spitzoid naevi and tumors, namely Tyrosine kinase fusions (B-RAF, ALK and RET fusions, singularly expressed, (Wiesner et al., 2014). Next, we will further modify each cell line, bearing the single fusions, for Ambra1 expression. Then, we will compare cell growth and aggressiveness of the resulting cell lines, by comparing each Ambra1-depleted line with the respective control cells (Melan-a expressing the Tyrosine kinase fusion only). To this aim, we will consider both *in vitro* and *in vivo* experimental models to work with (see methods below).
*Methods:* Melan-a cells will be transduced with lentiviral particles encoding the Tyrosine kinase fusions (already available in the literature, (Wiesner et al., 2014) and a Doxycycline-inducible small-interference RNA targeting Ambra1, already available and successfully used in our lab. A non-targeting doxycycline-inducible small-interference RNA will also be used as a negative control.
As for the *in vitro* assays, we will perform cell counting and MTT assay to assess cell proliferation, and transwell migration assay and soft-agar colony formation assay to test

whether Ambra1 can impact on aggressiveness and malignant transformation.
We will also use the transduced Melan-a cells, depleted or not for Ambra1, in a xenograft experiment. In particular, transduced cells will be injected subcutaneously and bilaterally into the flanks of immunocompromised mice. We will induce Ambra1 depletion by administration of doxycycline and monitor the mice for tumour formation every 3–4 days. In agreement with what previously observed (Wiesner et al., 2014), within 40 days the injection sites in the fusion kinase groups will develop rapidly growing tumors. Their development and size will be compared in sites injected with fusion kinase groups and Ambra1-depleted fusion kinase groups. The resulting tumors will be collected 40 days after injection and analysed by IHC with relevant markers: Ki-67 (cell proliferation marker), cyclin D1, p21, p27 (altered in spitzoid tumors and Ambra1 targets – see preliminary results above), HMB-45 (melanocytic tumor marker). Also, a histopathological analysis of the tissue samples will be performed (i.e. H&E staining) and interpreted by NN in our team.
Importantly, cell culture, transduction with Tyrosine kinase fusions, and xenografts with Melan-a cells were already demonstrated to represent successful approaches in the literature (Wiesner et al., 2014).

**Task 3. Characterization of AMBRA1 and cyclin D1, p21 and p27 interplay in the etiology of spitzoid tumors**

*Rationale:* While cyclin D1, p27 and p21 levels are often altered in spitzoid tumors, they do not seem to impact cell proliferation (Figure 2, unpublished data). This evidence raises the question whether/how their levels are relevant or not to the etiology and biology of spitzoid tumors. Also, the reason for the alteration of their protein levels (often not justified by genetic amplification) is completely unknown. Here we want to test the effect of increased cyclin D1/p21/p27 in a spitzoid tumor model, and to assess the possible function of Ambra1 as the master-regulator of cyclin D1/p21/p27 in spitzoid tumors.
*Experimental Plan:* We will use Melan-a cells transduced for the expression of Tyrosine kinase fusions (see also Experimenatal Plan in Task 2) and combine it with the doxycycline inducible over-expression of cyclin D1, p21 and p27. GFP expression will be used as a negative control. The resulting cell lines will be assessed for cell proliferation and cell malignancy/aggressiveness in *in vitro* and *in vivo* assays, as described in the Methods in Task 2 above.
In addition, we will assess the contribution of the Ambra1-mediated regulation of cyclin D1 to the aforementioned spitzoid phenotypes. To this end, we will silence the endogenous expression of Ambra1 and replace it with a mutant uncapable of regulating cyclin D1 (AMBRA1 ΔWD40, see also Preliminary Results above). Also, in this case, we will perform *in vitro* and *in vivo* experiments, as described in the Methods in Task 2 above.
*Methods:* Lentiviral plasmids encoding for the inducible over-expression of cyclin D1 and GFP are already available in our laboratory and have been successfully used. The same backbone plasmid will be used for cloning p21 and p27 cDNAs. Lentiviral plasmids encoding for wild-type and ΔWD40 mutant of AMBRA1 are available in our laboratory and have been already used with success. All other reagents and methods are as already described in the Methods section in Task 2 above.

## 8. PITFALLS and ALTERNATIVE STRATEGIES

*Relative to Task 1.* The antibody (Ab) against human AMBRA1 to be used on spitzoid tumors is already available in our lab and has been successfully used in IHC on FPPE sections of human brain. Also, we know that AMBRA1 is expressed in human skin, hence we do not foresee any technical issue with this Ab being used on spitzoid tumors. Regardless, in the case we will have any doubts on its reliability, we will perform a Fluorescence in situ hybridization (FISH) instead, by using appropriate probes (Fimia et al., 2007) and taking advantage of the already established protocol currently used in the histology core facility of the Bambino Gesù Children's Hospital (our Host Institution).
*Relative to Task 2 and 3.* As specified in the Methods section, Melan-a cells have been

successfully used for an experimental set-up very similar to ours. Hence, we do not foresee any problems with their usage. However, in the case of unexpected difficulties, we will use primary cells derived from spitzoid tumors. These cell lines have been already established, characterised and stored by our team member (NN). The cells can be transduced and grown in culture for few passages and in xenografts. Hence, they would be a useful model for our aims. Ethical permissions relative to these cells are as described in Addendum B. Briefly, they are already included in an approved ethical protocol (CE n. 8391/2013 Roma; S. Andrea Ethical Committee. Our team member NN is in the ethical protocol).

## 9. EXPECTED RESULTS AND RELEVANT CORRESPONDING MILESTONES

The timeline for the experimental plan is as follows:

| Task | Months 1-3 | Months 4-6 | Months 7-9 | Months 10-12 |
|---|---|---|---|---|
| 1 | X | X M1 | | |
| 2 | X | X M2 | | |
| 3 | | | X | X M3 |

Expected results and relevant corresponding milestones are as follows:
*Milestone 1* (**M1**) Validation of AMBRA1 as a novel diagnostic biomarker for spitzoid tumors, to be used in combination with cyclin D1, p21 and p27.
*Milestone 2* (**M2**) Identification of AMBRA1 as a tumor suppressor in spitzoid tumors.
*Milestone 3* (**M3**) Characterization of the interplay among AMBRA1, cyclin D1, p21 and p27 in the etiology of spitzoid tumors.

Altogether, if successful this proposal will: i) improve the challenging diagnosis of spitzoid tumors; ii) provide new interpretation for the conflicting role of cyclin D1, p21 and p27 in spitzoid tumors etiology and diagnosis, iii) characterise, for the first time, the role of the autophagy and cell cycle regulator AMBRA1 in a melanocytic tumor.

## 10. REFERENCES and RELEVANT PUBLICATIONS BY THE RESEARCH GROUP, ALREADY AVAILABLE in red


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- *Nagasaka, T., Lai, R., Medeiros, L.J., Brynes, R.K., McCourty, A., Harada, T., and Saddik, M. (1999). Cyclin D1 overexpression in Spitz nevi: an immunohistochemical study. Am. J. Dermatopathol. 21, 115–120.*
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## PERSONNEL INVOLVED IN THE RESEARCH

| Name and date of birth | Role on Project | Fellowship required | Effort on project (%) | Present position |
|---|---|---|---|---|
| Valentina Cianfanelli (**VC**) 09/02/1985 | Principal Investigator | NO | 30% | Post-Doctoral Research Fellow |
| Giacomo Milletti (**GM**) 28/08/1994 | PhD Student | NO | 30% | PhD Student |
| Roberto Cirombella (**RC**) 13/05/1978 | Lab technician | NO | 30% | Lab technician |
| Niccolo Noccioli (**NN**) 09/10/1993 | Anatomical Pathology Specialist | NO | 40% | Specialist Medical Student in Anatomical Pathology |
| Caterina Ferraina (**CF**) 18/03/1979 | Lab technician | YES | 33% | Lab Technician |
| To be determined (**TBD** - young researcher) | Post-Doctoral Fellow | YES | 100% | Young Post-Doctoral Research Fellow |

## DESCRIPTION OF THE WORK FOR EVERY UNIT OF PERSONNEL

1) **VC** (PI) will coordinate and supervise the research and project activities, design the experimental plan, analyse data and interpret the results
2) **GM** (PhD Student. Same affiliation as for the PI) will produce the lentiviruses for cell transduction (Task 2 and 3)
3) **RC** (Lab technician. Affiliation: Pathology Research Lab, Sant'Andrea University Hospital, via di Grottarossa 1035, 00289 Rome, Italy) will perform the IHC on patient samples (Task 1, 2 and 3)
4) **NN** (Specialist Medical Student. Affiliation: Pathology Research Lab, Sant'Andrea University Hospital, via di Grottarossa 1035, 00289 Rome, Italy) will interpret the results of the immunohistopatological assays on patient samples and xenografts (Task 1, 2 and 3)
5) **CF** (Lab technician. Same affiliation as for the PI) will assist with the molecular and biochemical assays (protein extraction, plasmid amplification, immunoblottings) for 33% of her time
6) **TBD** (Young Post-Doctoral Research Fellow) will establish the melanocytic cell lines and perform the related experiments (Task 2 and 3). Also, he/she will also partecipate to the set-up AMBRA1 IHC on patient samples (Task 1)

Budget Form /year

1. research costs **€ 27.000**
2. Instruments **€ 0**
3. Indirect costs **€ 7.000**
4. **Sub-total 34.000**
5. Overheads (10%) **€ 6.200**

6. Fellowships **€ 28.000**
7. **Total 68.200**

Justifications Itemized research costs

**Research costs:** Antibodies and reagents for IHC; reagents for cell culture, lentiviral production and cell transduction; reagents for biochemical and molecular assays (cloning, western blots, plasmid amplification), reagents for cell biology assays (MTT, trans-well migration assay) general consumables, *in vivo* experiments (xenograft injection) performed at the Castel Romano S.r.l. animal facility

**Indirect costs:** pubblications on international journals, partecipation to an international conference/meeting for one unit personnel

**Fellowships:** One Young Post-Doctoral Research Fellow (**TBD**, 100% effort: € 22.000) and (**CF**, 33% effort: € 6.000)

**EXISTING/PENDING SUPPORT NO**

**SUGGESTED REVIEWERS (MAX 3)**

1) **Dr. Paolo Ascierto**
Istituto Nazionale Tumori Fondazione G. Pascale, via Mariano Semmola, 53, 80131 Naples, Italy
e-mail: p.ascierto@istitutotumori.na.it

2) **Dr. Michele Milella**
IFO – Istituto Nazionale Tumori Regina Elena, via Elio Chianesi 53, 00144 Rome, Italy
e-mail: michele.milella@aovr.veneto.it

3) **Leonard Girnita**
Dept. Oncology Pathology, Cancer Center Karolinska, Karolinska Hospital, Stockholm, Sweden
e-mail: leonard.girnita@ki.se

**BIOETHICAL REQUIREMENT**

1. Human experimentation YES – please provide clearance from the competent ethical committee as addendum A

2. Animal experimentation YES – please include a statement as addendum B specifying which regulations the proposed research meets

**Declaration**

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

Date: 15/01/2021 Name of PI **Valentina Cianfanelli** signature

**Principal investigator's signature Valentina Cianfanelli**

**Authorized Administrative Official's signature Prof. Dallapiccola**

Il Direttore Scientifico
(Prof. Bruno Dallapiccola)

Date 15/01/2021

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