The undersigned

SURNAME Broccoli **FIRST NAME** Vania, **born in** *Cesena* Prov. *FC* on 08/09/1969
Unit: *Stem Cell and Neurogenesis, Division of Neuroscience.*
Email address: *broccoli.vania@hsr.it*

Role:

☐ Vita-Salute San Raffaele University Professor/Lecturer

☐ Vita-Salute San Raffaele University Researcher/Lecturer

X Group Leader of the hospital site _____

☐ Project Leader of the hospital site _____

☐ Other

I hereby declare that, within the framework of the PhD Course for which I wish to submit the project described below:

X I am already a Supervisor;
☐ I am applying for the first time as a Supervisor (CV attached);
☐ I am applying as a Supervisor as three years have elapsed since my last Application as Supervisor and the submission of a research project (CV attached).

I further declare that (select the applicable option(s)):

X although I am less than four years away from retirement as a university professor/researcher, I will hold a documented institutional role at the hospital _____, for at least one year beyond the official duration of the course.

X I serve as Supervisor for no. 1 PhD candidates enrolled at other universities and I comply with the University requirement regarding the maximum number of five PhD candidates that may be supervised.

I would like to present a project:

X With a duration of three years
☐ With a duration of two years within the Physician Scientist (PhS) programme

as part of the PhD course in:

☐ Molecular Medicine

PhD Curriculum:

☐ Basic and Applied Immunology and Oncology

☐ Cell and Molecular Biology

☐ Clinical and Experimental Medicine

☐ Neurosciences and Experimental Neurology

X Gene and Cell Therapy

☐ Cognitive and Behavioural Sciences

The project consists in:

1. Basic Research ☐
2. Translational Research X
3. Basic/ Translational research using animal models X
4. Clinical research ☐
5. Clinical research involving interaction with patients ☐

If items 2 and/or 3 is/are selected, I declare that

X I HAVE OBTAINED the approval of the responsible Institutional Animal Care and Use Committee-IACUC number: 1636

☐ I HAVE NOT YET OBTAINED the approval of the responsible Institutional Animal Care and Use Committee-IACUC

If items 4 and/or 5 is/are selected, I declare that the project:

o **HAS NOT YET OBTAINED** approval from the Ethics Committee (EC)

o **HAS OBTAINED**, or is part of a broader study that has obtained, approval from the Ethics Committee (EC); study code and date _____

If items 4 and/or 5 is/are selected, I declare that the project:

☐ **HAS NOT OBTAINED** the resolution of the Institution

☐ **HAS OBTAINED** the resolution of the Institution on _____

I further declare (select the applicable option(s)):

☐ that I have the availability of the funds necessary to finance a scholarship for the proposed project and I confirm that I have contacted the Doctoral Office regarding the management of the administrative procedures related to the funding;

X that I have the availability of funds to support the research (i.e. funds for materials, reagents, and instruments required for research activities);

☐ that, in the case of a clinical research project, it will include a basic or translational research component to be carried out in a laboratory to be specified in the research plan, whose head will act as co-supervisor.

X that I have adequate workspace and a permanent workstation available for the PhD candidate who will be selected to carry out the project;

X that the proposed project can be reasonably completed within the three-year legal duration of the programme;

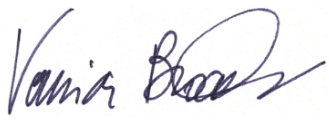
X that the PhD student, within the activities of the relevant PhD program, will carry out only their specific doctoral project;

X that the PhD student will be the first author/author of the main publication resulting from his/her project and of all publications (also after graduation) that are mainly based on his/her experimental work;

X that in the event that a student is not a recipient of a UniSR (i.e. has won a position without a grant), I am prepared to cover his/her grant.

X that, in the event that the PhD student is not the recipient of a UniSR grant (i.e. has won a position without a grant), I am willing to cover the cost of their scholarship with funds at my disposal. I am aware that the grant must not amount to less than the minimum required by the Ministerial Decree of 23 February 2022, amounting to € 16,243 gross per year, for three years;

☐ that the study is co-funded by an industrial partner or that a commercial exploitation of the findings resulting from the project's research activity is conceivable, with a potential delay in the publication of the results. I therefore commit to promptly inform potential candidates of such circumstances.

Signature of the Supervisor _____  ____ *Date: 31/03/2026*

<u>When applicable:</u>

Group Leader Prof. /Dr. _____

Signature _____ *Date*

Please note that the information provided on the following pages (unless otherwise indicated) will be made public on the University website. Therefore, it is important not to include confidential information, in compliance with any confidentiality obligations towards third parties and to protect the potential patenting of such information. For any questions, please consult the PhD Office.

PROJECT

Supervisor:	VANIA BROCCOLI
Title:	ENGINEERING AAV-BASED THERAPEUTIC STRATEGY FOR PRECISE NEURAL TUMOR TREATMENTS
Curriculum:	Gene and Cell Therapy
Link to the personal page of the University or relevant hospital site website:	https://research.hsr.it/en/divisions/neuroscience/stem-cells-and-neurogenesis/vania-broccoli.html

Description of the Project (max 3,000 characters including spaces)

Background/gap of knowledge

Adeno-associated viral (AAV) vectors are widely used in gene therapy due to their versatility and favorable safety profile. However, their broad tropism limits their suitability for cell-specific applications. Current AAV variants often exhibit off-target transduction across multiple organs and cell types, reducing overall efficiency and raising potential safety concerns. These limitations hinder the use of AAVs in precision medicine approaches that require strict cell-type specificity, such as the targeted delivery of therapeutics to primary or metastatic tumor cells.

Rationale and hypothesis

We developed AAV-STITCH, a strategy using SpyTag technology (1) to covalently attach polypeptides to the AAV capsid. This system relies on two small reactive protein fragments, SpyTag and SpyCatcher, which spontaneously form a covalent isopeptide bond with high affinity. Given that the SpyTag fragment consists of only 13 amino acids, we integrated it into the AAV capsid maintaining intact the production efficiency of this vector. As proof of concept, we fused the SpyCatcher with the anti-GD2 ScFv to redirect its infection to GD2-expressing neuroblastoma (NB) cells (2). NB is the most frequent and aggressive pediatric extracranial solid tumor, responsible for 15% of childhood cancer deaths. AAV-STITCHαGD2 selectively transduced NB tumor cells without transduction of healthy tissues. Furthermore, delivery of a suicide gene via AAV-STITCHαGD2 suppressed tumor growth and extended survival in mice with subcutaneous and pseudometastatic NB xenografts (2). These findings establish the feasibility of engineering AAVs with cell-type-specific transduction properties, providing a powerful and adaptable platform for the selective elimination of NB tumor cells.

Objectives and specific aims

Our data support the strong therapeutic potential of the AAV-STITCH-GD2 system, representing a radical new and powerful strategy to fight NB and other GD2+ tumors. In this project, this approach will be tested in multiple pre-clinical models of NB and glioblastoma and further evolve the system with more potent viral vectors able to maximize tumor killing and evade host immune system. The AAV-STITCH-GD2 will be further evolved by: i) identifying the most effective suicide gene able to also stimulate the host immune system in a NB congeneic mouse model; ii) extending its application to GD2+ NB brain metastasis and glioblastoma; iii) overcoming its current limitation as one-single treatment by enabling repeated AAV dosing.

Expected outcomes

This project will optimize and advance a groundbreaking therapeutic approach by engineering AAV capsids to precisely target NB tumor cells with unprecedented specificity and efficacy, establishing a new paradigm in precision medicine.

Skills that the student should acquire (max. 600 characters including spaces):

The student will develop strong expertise in:
- Molecular cloning and gene engineering
- AAV vector cloning, production and quality assessment
- Manipulation and analysis of mouse models of Neuroblastoma and Glioblastoma
- Brain tissue isolation and histological analysis
- Genome-wide bulk and single-cell transcriptomics studies
- Basic knowledge of bioinformatics tools
- Immunofluorescence and image analysis
- Statistics and data interpretation

References (max. 15)

1) Zakeri B, et al. Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proc Natl Acad Sci U S A.* **109**, E690-7 (2012).

2) Luoni M, Giannelli G. S., parracino S, Morosi F, Ventura E, Di Pizio A, Muggeo S, Iannielli A, Canu T, Russo T., Esposito A., Pastorino F., Broccoli V. targeting and efficient AAV therapy for neuroblastoma via direct capsid-antibody coupling. bioRxiv 2025.11.12.687993; https://doi.org/10.1101/2025.11.12.687993.

The information below will not be displayed on the University website in the description of the projects offered for the academic year, and will be used for internal project assessment only.

Experimental plan (Between 2,000 and 3,000 characters including spaces):

To be completed for all types of projects; however, for CLINICAL PROJECTS, please specify:

1. *If Observational prospective, cross-sectional, or retrospective) or retro/prospective, quality of life, pharmacological, pathophysiology, genetics, epidemiological, registry/data collection, biobank, diagnostic accuracy, in vitro diagnostic device (IVD), nutraceutical/supplement, appropriateness; OR interventional (pharmacological, surgical, procedure, or medical device, and if a drug will be used, indicate the phase – I, II, III, or IV);*
2. *If a drug will be used, specify whether it has a marketing authorisation (MA), whether it will be used according to the MA or whether it does not have a MA;*
3. *If the study does not regard a drug, specify what will be studied (e.g. medical device, surgical procedure, diagnostic procedure, food supplement, etc.). If the study will use a medical device, please specify: whether it is CE marked. If CE marked, please indicate whether it will be used according to the approved use or for a new use.*
4. *Indicate the laboratory on which you intend to rely for the basic or translational part.*

The project is articulated in three main tasks:

T1) Assess antitumor effects of TK-expressing AAV-STITCH-aGD2 vector in a syngeneic NB immunocompetent mouse model. High-risk NBs are cold and non-immunogenic tumors, with low expression of surface MHC class I molecules and consequently low neoantigen presentation. However, activated tumor infiltrating lymphocytes have been reported and their presence appears to correlate favorably with clinical outcome. Thus, activation of the immune system might elicit an important antitumor action. Our previous studies with the AAV-STITCH-aGD2 were carried out in immunodeficient animals preventing to assess this important aspect. This project will investigate this aspect by testing the AAV-STITCH-aGD2 therapy in a syngeneic immunocompetent NB mouse model. For this study, the 9464D-GD2 cell line will be employed, which is derived from a spontaneous neuroblastoma developed in a TH-MYCN transgenic mouse on the C57BL/6 background. 9464D-derived tumors have similar characteristics to human high-risk NB, with a low tumor mutation burden and MYCN overexpression. GFP-Luc2-9464D-GD2 cells (2x10e6) will be injected subcutaneously onto the flank of C57BL/6 mice. 7 days later, animals will be left untreated or treated with AAV-STITCH-aGD2

expressing HSV-TK or mCherry (control). Ganciclovir will be administrated daily for the following 7 days in all three groups of mice. Tumor growth dynamics will be monitored by IVIS optical imaging over time. This task will reveal the interplay between HSV-TK expressing NB tumor cells and the host immune system and its contribution in the final antitumor effects of this therapy.

T2) Compare GSDMD-N- or TK-expressing AAV-STITCH-aGD2 in syngeneic and patient derived xenograft (PDX) NB mouse models. The project will assess the therapeutic potential of GSDMD-N-induced pyroptosis in NB tumor cells promoted by the AAV-STITCH-aGD2. A syngeneic immunocompetent NB mouse model will be employed with subcutaneous grafts of GFP-Luc2-9464D-GD2 cells. The project will compare the antitumor effects of both GSDMD-N or TK expressing AAV-STITCH-aGD2 systems with equal viral dose of 5x10e10 vg/mouse respect to untreated animals (3 experimental groups). To enhance their clinical value, both viral vectors will be tested in a PDX mouse model. Patient tumor samples are obtained from the Alex's Lemonade Stand Childhood Cancer Repository (*https://www.cccells.org/index.php*) amplified through consecutive transplantations into mice.

T3) Engineering AAV capsids to blunt immune response and allow repeated viral dosing. Activation of the innate immune system represents the first event that mounts the immunity against AAV after in vivo administration Thus, the project will employ the STITCH system to decorate the AAV capsid with the mouse extracellular CD47 region. CD47 will be fused with the SpyCatcher and link to the AAV9 mutant capsid carrying the SpyTag domain. Viral production yield and coupling efficiency will be assessed by viral genome analysis and Western blot against CD47 and VP viral proteins as shown in preliminary data. Effective protection from phagocytosis will be initially tested using the murine macrophage-like cell line RAW 264.7. Phagocytosis of AAV particles will be assessed by viral genome quantification in cellular lysates. Next, the best performing engineered AAVs will be inoculated intravenously in immunocompetent mice and serum levels of inflammatory cytokines will be scored and compared to those elicited by standard uncoupled AAVs. The transduction efficiencies of GFP+ hepatic metastatic neuroblastoma cells with single or dual viral infection will determine the relative efficacy of the second infection.

Available methods and experimental models (max. 600 characters including spaces):

To be completed for all types of projects; however, for CLINICAL PROJECTS, please specify:

1. *whether participants (patients and/or healthy volunteers) will be recruited;*

2. *whether biological samples will be taken from participants (patients and/or healthy volunteers);*
3. *whether the biological samples will be stored in a Biobank (specify which Biobank);*
4. *whether biological samples are already stored and available in a Biobank (specify which Biobank);*
5. *whether biological samples or data will be collected in addition to those already included in the routine standard of care from routine practice (specify type of samples/data, quantity and timing);*
6. *whether procedures will be required in addition to those already included in the routine standard of care from routine practice (e.g. Consultations, laboratory tests, clinical/instrumental examinations). Specify the additional procedures, quantity and timing).*

AAV production is carried out with standard triple-transfection of HEK293T cells using AAV-producing plasmids and the SpyCatcher-αGD2 vector. We established the co-producing protocol with the virus and the SpyCatcher-αGD2 fusion expressed in the same cells, followed by purification on iodixanol gradient, resulted in the efficient removal of unbound SpyCatcher-αGD2, which was largely excluded from the final viral preparation (co-expression protocol). This project will employ the following Neuroblastoma mouse models:

Pseudometastatic model

SH-SY5Y cells were resuspended in sterile PBS at a concentration of 1x10e6 cells/ml and then 100 µl (100.000 cells) were injected into the tail vein of NSG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) using 0,3 ml syringes. The extent of liver metastasis was then asses through MRI imaging at 2- and 3-weeks post injection for metastatic burden evaluation.

Subcutaneous model

3x10e5 SH-SY5Y cells were seeded in a well of a 6 multi-well plate and transduced with LV-Luc-ires-Puro (10e8 IFU/ml). After 72h of puromycin selection, we expanded the cells in a T25 flask. SH-SY5Y-Luc+ cells were then resuspended in a 50% Matrigel (Matrigel growth factor reduced, Corning) solution in sterile PBS at a concentration of 2x10e7 cells/ml. Then 100 µl per mouse of solution were subcutaneously injected in the right flank of NSG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) using 0,3 ml syringes. Evaluation of tumor burden progression occurred through BLI imaging (IVIS spectrum) at multiple timepoints from the second week post injection onward trough intraperitoneal luciferin injection.

Pateint-derived Xenograft (PDX) model

Patient-derived cells were kindly donated by Childhood Cancer Repository (https://www.cccells.org/). 5x10e6 cells were injected subcutaneously in NSG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) using 0,3 ml syringes. After two weeks, mice were injected intravenously with AAV-STITCH-GD2 expressing Luciferase to evaluate tumor burden progression through BLI imaging (IVIS spectrum) at multiple timepoints onward trough intraperitoneal luciferin injection.

Role of the PhD student (max. 600 characters including spaces):

The PhD student will be introduced to molecular studies and mouse models by senior researchers in the lab who have extensive experience with these technologies. S/he will collaborate to analyze the Omics data with a bioinformatician in the lab who has developed solid skills in these genome-wide analyses. After this initial training, S/he will carry out the project under the direct supervision of the head of unit with whom s/he will have weekly meeting to discuss the planning, execution and interpretation of the ongoing experimental work.

Impact of the expected results in the field of research (max. 600 characters including spaces):

This project stems from the new and powerful AAV-STITCH technology that enables to reprogram AAV tropism exclusively to GD2+ NB tumor cells with no detectable off-targets in vitro and in vivo. This platform represents a novel paradigm based on suicide gene-expressing AAVs with a reprogrammable tropism specific for tumor cells. This strategy is radically different from the landscape of available antitumor therapeutics and can be easily combined with them. This strategy has the limitations that can be administrated only as single treatment in patients with no pre-existing AAV immunity. This project will elaborate new engineering strategies to armor the AAV capsids for minimizing these shortcomings and allow repeated viral dosing. Once fully established, this approach has the potential to develop into a powerful targeted cancer therapy.

In the case of clinical research, include the timeline for the project approval process up to the authorizing resolution of the Institution.

Period of attendance at a foreign institution

Mandatory for the PhD course in Cognitive and Behavioral Sciences

The PhD course in Cognitive and Behavioral Sciences encourages attendance at foreign universities and research institutes, promoting the acquisition of advanced skills and methodologies in international contexts.
Please indicate whether a period of activity at a foreign institution is planned. If so, specify:

- *Host institution (name of the University/Institute and country)*
- *Duration of stay (not less than 3 months)*
- *Integration with the research project (describe how this experience will contribute to the objectives of the proposed project)*

The information provided is not binding and may be subject to modifications based on the project's development and available opportunities.

For the use by the PhD Office

FOR OPINION – *(ONLY for Programs divided into Curricula)*

Signature of the Curriculum Supervisor _____ *Date* _____

FOR APPROVAL

Signature of the PhD Course Coordinator _____