The undersigned

SURNAME Squadrito **FIRST NAME** Mario Leonardo born in Argentina Prov. Bs As on 08 /09/1984

Unit: *Vector Engineering and In Vivo Tumor Targeting (SR-Tiget); Residency/Postgraduate School*[1]: _____-_____; *Email address*: *squadrito.mario@hsr.it*

Role:

☐ Vita-Salute San Raffaele University Professor/Lecturer

☐ Vita-Salute San Raffaele University Researcher/Lecturer

X Group Leader of the hospital site SR-Tiget

☐ 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)):

☐ 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. 3 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:

[1] To be indicated only for research projects associated with the Physician Scientist programme

X 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	☐
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

☐ I HAVE OBTAINED the approval of the responsible Institutional Animal Care and Use Committee-IACUC number _____

X 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)):

- x 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 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* ***30/03/2026***

<u>When applicable:</u>

Group Leader Dr. Mario Leonardo Squadrito - Signature _____ *Date* ***30/03/2026***

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*:**	Mario Leonardo Squadrito
***Title*:**	***Reprogramming antigen trafficking and immune responses using in vivo lentiviral gene therapy***
***Curriculum*:**	Gene and Cell Therapy
***Website*:**	https://research.hsr.it/en/institutes/san-raffaele-telethon-institute-for-gene-therapy/units/vector-engineering-and-in-vivo-tumor-targeting/mario-squadrito.html

Description of the Project

1. Background / Gap of Knowledge

In vivo gene therapy enables direct genetic modification of target cells using viral vectors, without the need for ex vivo cell manipulation [1,2]. Lentiviral vectors (LVs) are powerful platforms for stable, cell-type-specific gene delivery, increasingly explored for applications beyond monogenic diseases, including cancer immunotherapy and immune modulation [3]. However, a fundamental and underexplored question is how antigen expression driven by LVs shapes systemic immune responses depending on which cell type is targeted. The liver, a primary organ transduced by intravenously administered LVs, is an immunologically tolerogenic environment [4,5]. Whether hepatocyte-directed antigen expression induces dysfunction or deletion of antigen-specific T cells, and how this compares to antigen delivery to myeloid or other immune cell populations, remains poorly understood. Closing this gap is essential to both improving gene therapy outcomes and exploiting LVs as precision immune modulators.

2. Rationale and Hypothesis

We hypothesize that antigen trafficking and cellular targeting can be engineered using LVs to actively reprogram immune responses *in vivo*. Specifically, directing antigen expression to defined cell populations (e.g. hepatocytes vs. myeloid cells) will differentially shape T cell activation, dysfunction, or tolerance [6]. By leveraging vector engineering, LVs can be transformed from passive gene delivery tools into active modulators of immunity [7,8]. Understanding and controlling these processes will enable the design of therapies that either enhance immunity (e.g. anti-tumor responses) or induce tolerance (e.g. in chronic inflammation or autoimmunity).

3. Objectives and Specific Aims

Aim 1: Define how antigen trafficking and cellular targeting influence immune outcomes

- Engineer LVs with distinct cellular tropism and antigen presentation formats.
- Assess antigen uptake, processing, and MHC-I/II presentation *in vivo* across target cell populations.

Aim 2: Dissect how LV-driven antigen expression shapes adaptive immunity

- Characterize $CD8^+$ T cell activation, exhaustion, or deletion induced by cell-type-specific antigen delivery.
- Integrate single-cell transcriptomics and spatial profiling to map immune responses at tissue level.

Aim 3: Develop strategies to reprogram immune responses using engineered LVs

- Incorporate immunomodulatory payloads (cytokines, regulatory RNAs, co-stimulatory signals).
- Validate approaches to enhance anti-tumor immunity or reverse antigen-specific tolerance in preclinical models.

4. Expected Outcomes

This project will establish a mechanistic framework linking antigen trafficking, cellular targeting, and immune fate decisions in vivo. It is expected to:

- Reveal how distinct antigen delivery routes differentially shape T cell responses.
- Identify key molecular pathways governing immune activation versus tolerance.
- Generate engineered LV platforms with programmable immunological outputs.

Ultimately, this work will provide conceptual and technological tools to advance both gene therapy and immunotherapy, with relevance to cancers where immune suppression limits the efficacy of current treatments.

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

The student will acquire hands-on experience in molecular cloning and lentiviral vector production, including the engineering of cell-type-specific constructs. *In vivo* skills will include mouse handling, vector administration, and tumor models. On the immunology side, the student will learn multiparametric flow cytometry for T cell phenotyping, antigen presentation assays, and functional immune readouts. The project also incorporates single-cell RNA sequencing. Overall, the project offers a broad and modern training combining molecular biology, in vivo experimentation and immunology.

References (max. 15)

1. Kerzel, T., Giacca, G., Beretta, S., Bresesti, C., Notaro, M., Scotti, G.M., Balestrieri, C., Canu, T., Redegalli, M., Pedica, F., et al. (2023). In vivo macrophage engineering reshapes the tumor microenvironment leading to eradication of liver metastases. Cancer cell 41, 1892-1910 e1810. 10.1016/j.ccell.2023.09.014.
2. Milani, M., Fabiano, A., Perez-Rodriguez, M., Hernandez, R.J., Zecchillo, A., Zonari, E., Ottonello, S., Basso-Ricci, L., Canepari, C., Volpin, M., et al. (2025). In vivo haemopoietic stem cell gene therapy enabled by postnatal trafficking. Nature 643, 1097-1106. 10.1038/s41586-025-09070-3.
3. Giacca, G., Naldini, L., and Squadrito, M.L. (2024). Harnessing lentiviral vectors for in vivo gene therapy of liver metastases. Clin Transl Med 14, e1542. 10.1002/ctm2.1542.

4. Milani, M., Annoni, A., Moalli, F., Liu, T., Cesana, D., Calabria, A., Bartolaccini, S., Biffi, M., Russo, F., Visigalli, I., et al. (2019). Phagocytosis-shielded lentiviral vectors improve liver gene therapy in nonhuman primates. Science translational medicine 11. 10.1126/scitranslmed.aav7325.
5. Andreata, F., Laura, C., Rava, M., Krueger, C.C., Ficht, X., Kawashima, K., Beccaria, C.G., Moalli, F., Partini, B., Fumagalli, V., et al. (2024). Therapeutic potential of co-signaling receptor modulation in hepatitis B. Cell 187, 4078-4094 e4021. 10.1016/j.cell.2024.05.038.
6. Squadrito, M.L., Cianciaruso, C., Hansen, S.K., and De Palma, M. (2018). EVIR: chimeric receptors that enhance dendritic cell cross-dressing with tumor antigens. Nature methods. 10.1038/nmeth.4579.
7. Notaro, M., Borghetti, M., Bresesti, C., Giacca, G., Kerzel, T., Mercado, C.M., Beretta, S., Monti, M., Merelli, I., Iaia, S., et al. (2025). In vivo armed macrophages curb liver metastasis through tumor-reactive T-cell rejuvenation. Nature communications 16, 3471. 10.1038/s41467-025-58369-2.
8. Bresesti, C., Carito, E., Notaro, M., Giacca, G., Breggion, S., Kerzel, T., Mercado, C.M., Beretta, S., Monti, M., Merelli, I., et al. (2025). Reprogramming liver metastasis-associated macrophages toward an anti-tumoral phenotype through enforced miR-342 expression. Cell reports 44, 115592. 10.1016/j.celrep.2025.115592.

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.*

This is a basic/translational research project conducted entirely in the **Vector Engineering and In Vivo Tumor Targeting** Unit (***SR-Tiget***), with no clinical or interventional component.

The project will use engineered lentiviral vectors (LVs) to investigate how antigen trafficking and cellular targeting shape immune responses *in vivo*. LVs will be designed to direct antigen expression to defined cell populations, primarily hepatocytes and myeloid cells, using cell-type-specific promoters and envelope engineering strategies. Following *in vivo* administration in mouse models, antigen uptake, processing, and presentation will be characterized across target cell populations. The downstream fate of antigen-specific $CD8^+$ T cells will then be assessed, including activation, exhaustion, or deletion, depending on which cell type presents the antigen. Preclinical tumor models will be used to evaluate the functional impact on anti-tumor immunity.

Building on this mechanistic understanding, the project will develop LV constructs incorporating immunomodulatory payloads, including cytokines, regulatory RNAs, or co-stimulatory elements, to actively reprogram immune responses toward either enhanced immunity or antigen-specific tolerance.

Experimental approaches will include molecular cloning, LV production, multiparametric flow cytometry, single-cell RNA sequencing, and spatial transcriptomics.

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).*

This is a non-clinical project. Available methods include molecular cloning, LV production and titration, *in vivo* vector administration, and subcutaneous and metastatic tumor models. Immune responses will be characterized by multiparametric flow cytometry and functional T cell assays. Transcriptomic approaches include single-cell RNA sequencing and spatial transcriptomics, with bioinformatic analysis in R. All reagents, mouse strains, and core facilities required are already available in the laboratory.

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

The PhD student will be the primary driver of the project, taking responsibility for experimental design, execution, and data interpretation and analysis across all aims. This includes engineering and producing LV constructs, performing *in vivo* experiments in mouse models, and carrying out immunological and transcriptomic analyses. The student will contribute to data interpretation and manuscript preparation. The student will work in a collaborative and interdisciplinary environment, interacting with experts in gene therapy, immunology, and computational biology.

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

This project will establish a mechanistic framework for how antigen trafficking and cellular targeting shape immune responses *in vivo*, filling a critical gap in both gene therapy and cancer immunology. By demonstrating that LVs can be engineered to programmably modulate immunity, the project will provide new tools to enhance anti-tumor responses or induce

tolerance in inflammatory diseases. These findings have direct translational relevance, with potential to inform the design of next-generation *in vivo* gene therapy and immunotherapy strategies.

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

NA

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.

NA

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 _____