The undersigned

SURNAME Montini

FIRST NAME Eugenio born in ***New York*** Prov. **NY** on **09 / 02 / 1967**

Unit: *SR-Tiget, Safety of gene therapy and insertional mutagenesis research unit*

Residency/Postgraduate School[1]: ***NA***

Email address: ***montini.eugenio@hsr.it***

Role:

☐ Vita-Salute San Raffaele University Professor/Lecturer

☐ Vita-Salute San Raffaele University Researcher/Lecturer

X Group Leader of the hospital site San Raffaele Telethon Institute for Gene Therapy

☐ 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:

☐ I am already a Supervisor;

☐ I am applying for the first time as a Supervisor (CV attached);

X 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. 2** 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:

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

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:

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	**X**
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

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

X I <u>HAVE NOT YET OBTAINED the approval</u> of the responsible Institutional Animal Care and Use Committee-IACUC

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

☐ **<u>HAS NOT YET OBTAINED</u>** <u>approval from the Ethics Committee (EC)</u>

☐ **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;

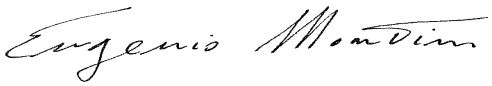
X that the PhD student, within the activities of the relevant PhD program, will carry out only their specific doctoral project;

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;

☐ 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.

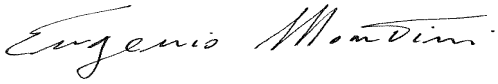
☐ that, in the event that the PhD student is not the recipient of a UniSR grant (i.e. has won a position without a grant), I am willing to cover the cost of their scholarship with funds at my disposal. I am aware that the grant must not amount to less than the minimum required by the Ministerial Decree 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 27/03/2026*

When applicable:

Group Leader Prof. /Dr. Eugenio Montini

Signature  *Date 27/03/2026*

PROJECT

Supervisor:	Eugenio Montini
Title:	***Decoding fitness and persistence of genome-edited human hematopoietic stem cells***
Curriculum:	Gene and Cell Therapy

Link to the personal page of the University or relevant hospital site website: http://research.hsr.it/en/institutes/san-raffaele-telethon-institute-for-gene-therapy/safety-of-gene-therapy-and-insertional-mutagenesis.html

Description of the Project

Background/gap of knowledge

Gene editing (GE) in hematopoietic stem and progenitor cells (HSPCs) enables precise correction of disease-causing mutations and is emerging as a transformative therapeutic strategy for genetic disorders[1]. In these approaches, patient-derived HSPCs are genetically modified ex vivo and subsequently transplanted to restore normal hematopoiesis after a conditioning treatment[2,3]. However, editing procedures often induce cellular stress responses, including cell-cycle arrest, senescence, and loss of stem-cell properties, that compromise HSPC fitness, leading to reduced engraftment efficiency and limited long-term persistence of edited clones[4,5]. These biological constraints remain a major obstacle for the broader clinical implementation of genome editing in hematopoietic stem cells.

Rationale and hypothesis

Long-term clinical follow-up of gene therapy patients has generated extensive datasets capturing the in vivo clonal dynamics of genetically modified HSPCs[6]. Analysis of these datasets reveals molecular programs associated with enhanced or impaired clonal persistence[7,8]. Based on these findings, we hypothesize that perturbation of specific regulators of stem cell fitness can improve the survival and long-term repopulation capacity of edited HSPCs without compromising genomic stability.

Objectives and specific aims

The project aims to identify and functionally validate cellular regulators that influence the fitness of edited HSPCs to improve their therapeutic performance.

A limited number (3–5) of candidate regulators will be prioritized for functional validation based on predefined criteria.

Specific aims:

1. Identify and prioritize candidate regulators of HSPC fitness through integrative analysis of clonal tracking datasets derived from gene therapy studies, to uncover molecular programs associated with clonal persistence and long-term contribution to hematopoiesis. Candidates will be selected based on clonal enrichment, reproducibility across datasets, and association with defined transcriptional programs.

2. Functionally interrogate selected regulators in human HSPCs by targeted perturbation using genome editing technologies, enabling direct assessment of their role in modulating stem cell fitness.

3. Evaluate the impact of these perturbations on editing efficiency, stem cell maintenance and hematopoietic differentiation potential.

Expected outcomes

This work will define molecular mechanisms that determine the fitness and persistence of edited hematopoietic stem cells. The results are expected to identify strategies to improve the efficiency, safety and durability of gene editing approaches. Ultimately, these findings may contribute to the development of more robust and clinically effective genome editing therapies for genetic diseases.

Skills that the student should acquire

The student will gain advanced training in human hematopoietic stem cell biology and genome editing. Skills include CRISPR-based perturbation, culture and functional analysis of human CD34+ stem/progenitor cells, flow cytometry, molecular assays, and clonal dynamics analysis via high-throughput sequencing. The student will also develop expertise in in vivo models of HSC function and engraftment, and in integrating experimental and computational approaches in a translational gene therapy context.

References

1. Naldini, L. Gene therapy returns to centre stage. Nature 526, 351–360 (2015).

2. Naldini, L. Genetic engineering of hematopoiesis: current stage of clinical translation and future perspectives. EMBO Mol. Med. 11, e9958 (2019).

3. Ferrari, S. et al. Genetic engineering meets hematopoietic stem cell biology for next-generation gene therapy. Cell Stem Cell 30, 549–570 (2023).

4. Schiroli, G. et al. Precise Gene Editing Preserves Hematopoietic Stem Cell Function following Transient p53-Mediated DNA Damage Response. Cell Stem Cell 24, 551-565.e8 (2019).

5. Conti, A. et al. Senescence and inflammation are unintended adverse consequences of CRISPR-Cas9/AAV6-mediated gene editing in hematopoietic stem cells. Cell Rep. Med. 6, 102157 (2025).

6. Six, E. et al. Clonal tracking in gene therapy patients reveals a diversity of human hematopoietic differentiation programs. Blood 135, 1219–1231 (2020).

7. Calabria, A. et al. Long-term lineage commitment in haematopoietic stem cell gene therapy. Nature 636, 162–171 (2024).

8. Scala, S. et al. Dynamics of genetically engineered hematopoietic stem and progenitor cells after autologous transplantation in humans. Nat. Med. 24, 1683–1690 (2018).

Experimental plan

The project will investigate cellular determinants that regulate the fitness of human hematopoietic stem and progenitor cells following genome editing. Candidate regulators will be prioritized through computational analysis of clonal tracking datasets derived from gene therapy studies in which genetically modified HSPCs have been monitored longitudinally in patients. These datasets provide a unique framework to relate the molecular profiles of individual HSPCs to their long-term in vivo behavior, based on the longitudinal tracking of genetically marked clones in patients. This approach enables the identification of regulators that influence clonal persistence, expansion, or functional exhaustion over time.

Selected regulators will be experimentally perturbed in primary human CD34+ HSPCs using genome editing approaches. Gene modulation strategies will be implemented using Cas9 ribonucleoprotein delivery in combination with AAV6-based donor templates or lentiviral vector systems, as appropriate for the specific experimental objectives, enabling controlled perturbation of target pathways. Editing cells will be perturbed with our selected regulators to preserve stem cell function while achieving efficient and reproducible genetic modification. These edited cells will be evaluated for editing efficiency, viability, proliferation, maintenance of stem cell phenotypes and functional progenitor activity.

A broad range of functional assays will be employed to assess the impact of these perturbations on key stem cell properties, including self-renewal capacity, multilineage differentiation potential and response to cellular stress. These analyses will be complemented by transcriptomic and molecular profiling to identify pathways and gene expression programs associated with improved cellular fitness and resilience to editing-induced stress.

Promising candidate regulators emerging from in vitro studies will be further evaluated in vivo using transplantation into immunodeficient mouse models to assess their impact on HSPC engraftment, clonal contribution and long-term repopulation capacity. Only top-performing candidates from in vitro assays will be advanced to in vivo validation to ensure feasibility within the PhD timeframe.

Together, these studies will provide a systematic framework to identify and validate molecular regulators that enhance the performance of genome-edited HSPCs, with potential implications for improving the efficacy of gene therapy strategies.

Human CD34+ HSPC isolation and culture, CRISPR-based genome editing, lentiviral vector systems, flow cytometry and molecular assays for stem cell characterization, high-throughput sequencing for clonal tracking analyses, and computational pipelines for analysis of clonal dynamics. Experimental systems include primary human hematopoietic stem cells and established functional assays for assessing stem cell maintenance and differentiation potential.

Role of the PhD student

The PhD student will perform experimental validation of regulators influencing the fitness of edited HSPCs. The student will design genome editing strategies, conduct experiments in primary CD34+ cells, analyse cellular phenotypes and integrate functional results with clonal tracking datasets. The student will participate in data interpretation, manuscript preparation and dissemination of results.

Impact of the expected results in the field of research

This project addresses a key limitation of genome editing therapies: the reduced fitness and persistence of edited hematopoietic stem cells. By identifying biological mechanisms that control stem cell fitness after editing, the project may provide new strategies to enhance the durability and efficacy of gene editing therapies. These advances could contribute to improving the treatment of severe genetic diseases currently targeted by hematopoietic stem cell gene therapy.

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

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 _____