TITLE: Antioxidant Treatment for Aging-associated Gut Inflammation (ATAGI)

PART 1 - PROJECT PROPOSAL

A. Abstract (to be provided also in Italian)

Inflammaging is a chronic low-grade systemic inflammation that occurs in elderly and promote several aging-associated diseases and dysfunctions, including an impaired intestinal regeneration. We found that anti-interferon treatment can mitigate this detrimental phenotype, however this approach is very expensive and not suitable as long-time therapy. Our preliminary data suggest that antioxidant treatment reduces intestinal inflammation in old mice. In this project we aim to test a novel and promising antioxidant molecule (NACET) to improve the aging-associated impairment of intestinal cellular homeostasis and regeneration. We expect to understand the molecular mechanism of drug action and to proof a systemic effect aimed to ameliorate organismal inflammaging. Our result will lay the basis for an effective, not invasive, cost-affordable approach against inflammaging-associated human dysfunctions and diseases.

Italiano: "Inflammaging" indica una infiammazione sistemica, cronica e di basso grado che compare durante l'invecchiamento ed è responsabile per una serie di malattie e disfunzioni associate alla vecchiaia, come per esempio una diminuita rigenerazione dell'apparato intestinale. In studi precedenti abbiamo trovato che i topi vecchi trattati con un anticorpo anti-interferone recuperano l'omeostasi e la capacità rigenerativa intestinale al livello dei topi giovani. Tuttavia, questo tipo di approccio ha poco valore clinico a causa dei potenziali costi elevati e degli effetti indesiderati di una possibile terapia permanente. Poiché l'infiammazione e lo stress ossidativo sono fortemente interconnessi nei tessuti invecchiati, abbiamo testato un antiossidante in topi vecchi. I risultati promettenti di questo esperimento pilota suggeriscono che questo trattamento potrebbe risolvere l'infiammazione intestinale tipica degli anziani. In questo progetto, noi vogliamo testare un nuovo trattamento antiossidante che è economico e non invasivo per capire se e come migliora l'omeostasi e la capacità rigenerativa intestinale, e se apporta benefici sistemici all'organismo.

B. Background and preliminary results

Aging is associated with a multitude of tissue dysfunctions and appearance of diseases, including several pathological conditions of the intestinal tract (impaired regeneration, diarrhea, malabsorption, cancer)[1,2]. It is long time known that aging is characterized by a chronic low-grade inflammatory state named inflammaging[3]. We recently found that inflammaging is driven by upregulation of innate immune receptors and systemic interferon gamma (IFNγ) signaling[4]. We have further demonstrated that the intestinal epithelium shows this pro-inflammatory phenotype during aging and that it is lost following long *in-vitro* culturing suggesting that is driven by external factors (e.g. the gut microbiome) and that can be reverted (opening therapeutics opportunity)[5,6].

Moreover, we found that treating mice with an antibody anti-IFNγ (Figure 1a) can revert the intestinal aging phenotype by rescuing the number of Lgr5+ intestinal stem cells (ISCs) and of Muc2+ Goblet cells (epithelial cells of the intestine responsible for the anti-microbial response) (Figure 1b) as well as the number of pro-inflammatory Cytotoxic Ccl5+ T-cells (Figure 1c) resident in the intestinal *lamina propria* to a young-like state. Importantly, pre-treatment with anti-IFNγ is able to improve the gut regeneration after treatment with the chemotherapeutic Fluorouracil (5FU) that induces intestinal damage as demonstrated by analysis of the mice body weight and histochemistry (Figure 1d-f) (Omrani, Krepelova et al, manuscript under revision). Overall, our data indicate that inhibition of IFNγ can have beneficial effects in elderly with intestinal pathologies and/or that undergo chemotherapy. Indeed, for some cancers (e.g. colon cancer), the chemotherapeutic drug cocktail has very severe side effects and, very often, drugs are combined according to the patient's health status. However, treatment with anti-IFNγ antibody has poor clinical value, since must be continuous and therefore becomes expensive and potentially leading to severe side effects, like reducing the immune defenses of the patients.

By employing Gene Ontology analysis of transcriptomes of intestinal epithelial cells isolated from old humans and mice, we found, together to inflammatory pathways, important signatures of oxidative stress enriched in aging organisms (Figure 1g). Oxidative stress can directly and indirectly induce pro-inflammatory signaling pathways that can in turn trigger a positive feedback loop by promoting oxidative

stress[7,8]. N-Acetylcysteine ethyl ester (NACET) is novel promising molecule with antioxidant activity, and with cell-permeable property much better than the well-known antioxidant N-Acetyl-L-Cysteine (NAC), whose recent clinical trials failed because of its low bioavailability[9,10]. We recently successfully tested NACET in old mice as potential treatment for age-related macular degeneration of the retina (data not shown). Beside this, we collected intestinal epithelial tissue from NACET-treated mice and performed some RT-qPCR analysis of specific epithelial markers and of target genes of IFNγ. We found that NACET-treated old mice rescued the expression of epithelial cell markers (Lrg5, Muc2) and IFNγ-target gene H2Ab1 (Figure 1h). These results, completely reflecting that one observed in old mice treated with anti-IFNγ, strongly suggest that NACET can mitigate the intestinal pro-inflammatory environment typical of the aged organisms and therefore potentially be a candidate for the treatment of aging-associated intestinal diseases and dysfunctions. In this proposal, we aim to better characterize the impact of NACET on improving aged gut functionality and ameliorating organismal inflammaging.

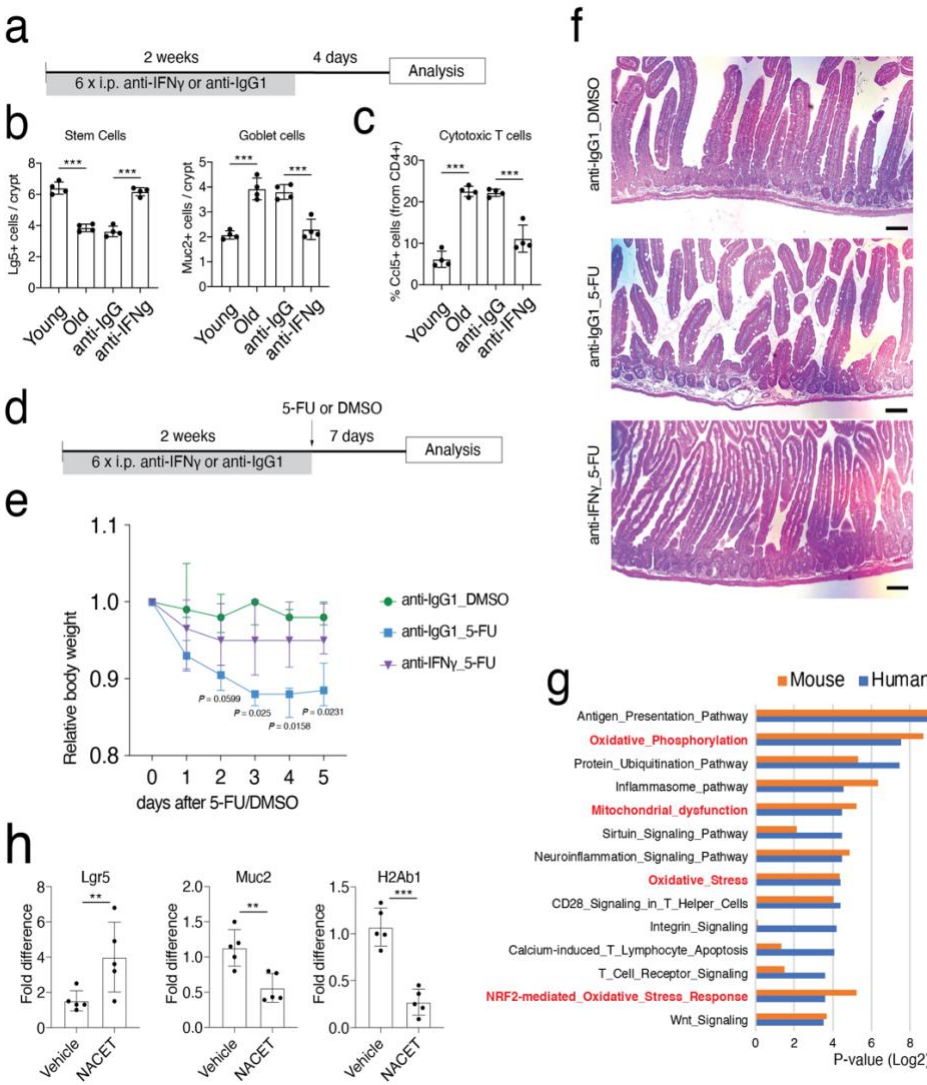

Figure 1: a) *Schematic representation of IFNγ inhibition experiment. Anti-IFNγ antibody was injected for two weeks (3 injections per week) in old mice. Mice were sacrificed for organ harvest 4 days after last injection. Anti-IgG1 antibody was the control.* ***b)*** *IF quantification of intestinal crypt old mice treated with anti-IgG (control) or anti-IFNγ. Young and old mice are also analyzed and shown as reference.* ***c)*** *FACS analysis quantification of Ccl5+ cells (from the CD4+ cells) isolated from the intestinal lamina propria of mice as in b).* ***d)*** *Schematic representation of the intestinal regeneration assay. Mice were treated as in a), but 1 day after last injection, 5FU or DMSO as control were injected.* ***e)*** *Total body weight of mice treated as in d).* ***f)*** *H&E-staining of longitudinal sections of the proximal part of the intestine from the indicated conditions. Scale bars 100μm.* ***g)*** *Gene ontology analysis of RNAseq of intestinal epithelium from young or old humans or mice. Pathways related to oxidative stress are highlighted in red.* ***h)*** *RT-qPCR of RNA isolated from intestinal crypts of old mice treated with vehicle (control) or NACET. Error bars represent the SD. P-value was calculated by Welch's t test (** p<0.01, ***p<0.005).*

C. Aims

In this project we aim to understand whether NACET treatment can: 1) ameliorate aging-associated gut phenotypes (inflammation, reduction of stem cells and increase of anti-microbic epithelial cells as Goblet cells) and for how long; 2) rescue aging-associated loss of intestinal regeneration. We want also to understand which are the pathways and gene targets of NACET in aged intestinal epithelial cells.

We found that brain from old mice shows transcriptional activation of the gene network proper of the inflammaging process[4] and that old mice and humans undergo detrimental gut dysbiosis in aging[11] and

data not shown). Therefore, we also aim to 3) understand the correlation of the intestinal inflammation with the neuroinflammation and the gut microbiome, specifically whether NACET treatment and consequent reduction of intestinal inflammation can also have an impact on brain inflammation and (at least partially) restore the microbial diversity of the gut microbiome to a more young-like state.

D. Project plan and eventual partners*

The project relies on 3 Working Packages (WPs) associated to three aims.

WP1: Characterization of the transcriptional response and cellular composition of the intestinal epithelium of old mice treated with NACET. We will employ single-cell RNAseq (10X genomics technology) that allows both analysis of variations of cell types and determination of the Differentially Expressed Genes (DEGs) for each cellular subpopulation. A separated old mice cohort will be treated for a week and analyzed after 2/4 weeks by IHC/IF analysis to assess the durability of treatment.

WP2: Characterization of the intestinal regeneration following 5FU treatment in old mice pre-treated with vehicle or NACET. Different groups of animals will pre-treated with NACET (dissolved in the drinking water) for 7 days before 5FU i.p. injection and then sacrificed after 1, 3 or 5 days from the 5FU treatment. Intestinal crypts will be collected for RNAseq and IHC/IF analysis. Mouse body weight will be measured every day to assess the intestinal regeneration.

DEGs obtained from the WP1 and WP2 will be analyzed by employing Gene Ontology and IPA software to find molecular pathways induced by NACET in physiological (WP1) or under regenerative (WP2) conditions. If timewise affordable, best target genes and pathways can be functionally tested in *in vitro* cultures of intestinal organoids by using shRNA or inhibitors.

WP3: Analysis of the neuroinflammation and gut microbiome. The brain and intestinal lumen content from WP1/WP2 mice will be collected for, respectively, RNAseq and 16s DNA sequencing to find potential connections between intestinal inflammation and neuroinflammation/gut microbiome. Results will be discussed with the neurologists Prof. Dr. Calvo (UniTo) and Prof. Dr. Vaz Patto (FCS/UBI, Beira, Portugal) and the gastroenterologist Prof. Dr. med. Settmacher (UKJ, Jena, Germany) for potential follow-ups and/or human translatability.

E. Expected results and contingency plans

From WP1, we expect to confirm our small pilot experiment (shown in Fig. 1h) and more precisely measure the changes at transcriptional and cell population level in the intestinal epithelium of old mice treated with NACET. In WP2 we suppose NACET treatment to restore the gut regenerative capacity of old mice to the level of the young ones. We also expect to understand if the molecular pathways induced by NACET in this regenerative context are the same of those induced in the more physiological context of the WP1. Overall, we want to deeply characterize the impact of an antioxidant treatment in the intestine of old mice. Specifically, we expect NACET to solve intestinal inflammation and to find its mechanism of action. Finally, we expect to understand whether solving the aging-associated oxidative stress can ameliorate systemic inflammaging and restore a "younger" gut microbiome. NACET is a novel molecule with anti-oxidative property as the currently-used NAC, but with improved pharmacokinetic features due to the esterification of the carboxyl group that increases both the lipophilicity and bioavailability of the compound. This study will elucidate clinical potential of the NACET in the treatment of aging- and inflammation-associated intestinal diseases.

Our group has experience in mouse experiment, genome-wide analysis as well as in aging and intestinal biology [4–6,12–15] thus the WP1 and WP2 should not show major technical barriers. If the *in vivo* data are not clear or some functional experiments are required, we can derive intestinal organoids from old mice and culture them in medium supplemented with pro-inflammatory cytokines, NACET and/or 5FU to mimic the *in vivo* conditions. For analysis of mouse brains, if necessary, we will collaborate with local colleagues (Prof. De Marchis, UniTo) or international partners (e.g. Prof. Wang, FLI, Jena or Prof. Studer, IBV, Nice) very expert in neuroscience. The most challenging part is the microbiome analysis in WP3 (high risk / high gain) because we do not have preliminary data and there are only few recent publications about antioxidant and gut microbiome relationship.

Remarkably, the three WPs are very connected each other, but negative results in one of them do not preclude the performance and success of the others.

PART 2 - PROPONENT'S CV

A1. Positions:

2020-to date Associate Professor, University of Torino, Italy
2016-2021 Group leader at the Leibniz Institute on Aging, Jena, Germany
2015-2016 Research Assistant, at the Radboud University MC, Nijmegen, Netherlands
2011-2015 Postdoc Fellow, Epigenetics unit, Human Genetics Foundation, Torino, Italy
2007-2011 PhD in Biotechnology, University of Siena, Italy

A2. Honors and other responsibilities

2021-to date Member of the Società Italiana di Biofisica e Biologia Molecolare (SIBBM)
2020-to date Member of the Board of the German Stem Cell Network (GSCN)
2017-2020 Member of the Internal Council of the FLI institute, Jena (Germany)
2017 GSCN 2017 Young Investigator Award
2016-to date Member of the German Foundation for Aging Research (DGfA)
2016 Sofja Kovalevskaja Award (on aging-associated epigenetic alterations)
2015 EMBO Short Term Fellowship Award

B. Publications *(metrics from WoS IF 2021)*

Total number of publications: 54 (H-index = 22)
Publications in the last 5 years: 29 (average IF = 12.2);
Publications in the last 5 years as corresponding author: 9 (aver. IF = 20.3)
5 most relevant publications in the last 5 years (***** Corresponding Author)

1. Lu, J., Annunziata, F., Sirvinskas, D., Omrani, O., Li, H., Rasa, S.M.M., Krepelova, A., Adam, L., and **Neri, F.*** (2022). Establishment and evaluation of module-based immune-associated gene signature to predict overall survival in patients of colon adenocarcinoma. J Biomed Sci 29, 81. **IF=12.771**
2. Rasa, S.M.M., Annunziata, F., Krepelova, A., Nunna, S., Omrani, O., Gebert, N., Adam, L., Käppel, S., Höhn, S., Donati, G., Jurkowsky, T.P., Rudolph, K.L., Ori, A., **Neri, F.*** (2022). Inflammaging is driven by upregulation of innate immune receptors and systemic interferon signaling and is ameliorated by dietary restriction. Cell Reports 39, 111017. **IF=9.995**
3. Freter, R., Falletta, P., Omrani, O., Rasa, M., Herbert, K., Annunziata, F., Minetti, A., Krepelova, A., Adam, L., Käppel, S., Rüdiger, T., Wang, ZQ., Goding, C., and **Neri, F.*** (2021). Establishment of a fluorescent reporter of RNA-polymerase II activity to identify dormant cells. Nature Communications 12, 3318–16. **IF=17.694**
4. Chen, Z., Amro, E. M., Becker, F., Hölzer, M., Rasa, S. M. M., Njeru, S. N., … , **Neri, F** et al. (2019). Cohesin-mediated NF-κB signaling limits hematopoietic stem cell self-renewal in aging and inflammation. The Journal of Experimental Medicine, 216(1), 152–175. **IF=17.579**
5. Ermolaeva M. *, **Neri F. *,** Ori A. *, Rudolph K.L. * (2018). Cellular and epigenetic drivers of stem cell ageing. Nature Reviews Molecular Cell Biology, 20(Suppl. 1), 667. **IF=113.915**

C. Conferences attended as invited speaker in the last 5 years

2018, Jena Aging Meeting JAM, Jena (Germany)
2019, Chromatin Club Symposium, Giessen (Germany)
2022, IMB CONFERENCE Epigenetics of Ageing, Mainz (Germany)

D. Current and anticipated grant support

Running: - AIRC MFAG, 2022-27: Origin of the aging-associated intestinal epigenetic drift and its impact in cancer development; - PNRR CN3 Spoke3 2022-24: Role of epi-modification of RNAs in neurodegenerative diseases
Submitted: CINA_MOST International Cooperation: Functional role of DNA methylation in modulating immunological response in the renal transplantation failure

E. Previous experience in collaborative research

I worked as postdoc at the HuGeF (Torino) on stem cells, differentiation and cancer where I collaborated with different local, national and international researchers. Then I led a Research Group of Epigenetics of Aging at the Leibniz Institute on Aging Research (Jena, Germany) where I collaborated with several European research groups and clinicians on aging-related diseases including cancer and adult stem cells dysfunctions and also acquired funding from Collaborative Grants (RTG1715; SAW-DRFZ2018; DFG international collaboration grant 2019). In Italy, I have submitted a Collaborative Grant (CINA_MOST) with Chinese clinical partner and a UniTo Grant for Internalization with a Portuguese clinical partner to study gut-brain axis in human aging.

F. Team

The PI Prof. Neri will supervise the project (experimental plan), train the PhD students, discuss the results, perform troubleshooting if needed, be responsible for manuscript writing. He will be also involved in the bioinformatic analysis (both performing and supervising). A predoc Fellow researcher will be employed for sample preparation for NGS sequencing and for data analysis with the supervision of the PI. A postdoc Fellow researcher with experience on animal work will be dedicated to mouse experiments and IF/IHC as well as to *in vitro* functional experiments. In addition, Dr. Daniela Donna, PhD and Dr. Anna Krepelova, PhD are experienced technicians of UniTo which can dedicate up to 15% of their time for the accomplishment of this project for all the 2 years. They will train PhD students in standard molecular biology techniques such as RNA/DNA purification and quality assessment. In addition, they will take care of administrative tasks as ordering of reagents and consumables, laboratory organization and databases, managing interactions with UniTo facilities (e.g. Sequencing Platform for NGS experiments).

References:

1. Bhutto, A. & Morley, J. E. The clinical significance of gastrointestinal changes with aging. *Current Opinion in Clinical Nutrition and Metabolic Care* 11, 651–660 (2008).
2. Pentinmikko, N. *et al.* Notum produced by Paneth cells attenuates regeneration of aged intestinal epithelium. *Nature* 571, 398–402 (2019).
3. Franceschi, C. *et al.* Inflamm-aging: An Evolutionary Perspective on Immunosenescence. *Annals of the New York Academy of Sciences* 908, 244–254 (2000).
4. Rasa, S. M. M. *et al.* Inflammaging is driven by upregulation of innate immune receptors and systemic interferon signaling and is ameliorated by dietary restriction. *Cell Reports* 39, 111017 (2022).
5. Lu, J. *et al.* Characterization of an in vitro 3D intestinal organoid model by using massive RNAseq-based transcriptome profiling. *Sci Rep-uk* 11, 16668 (2021).
6. Širvinskas, D. *et al.* Single-cell atlas of the aging mouse colon. *Iscience* 25, 104202 (2022).
7. Zuo, L. *et al.* Inflammaging and Oxidative Stress in Human Diseases: From Molecular Mechanisms to Novel Treatments. *Int J Mol Sci* 20, 4472 (2019).
8. Reuter, S., Gupta, S. C., Chaturvedi, M. M. & Aggarwal, B. B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Bio Med* 49, 1603–1616 (2010).
9. Giustarini, D., Milzani, A., Dalle-Donne, I., Tsikas, D. & Rossi, R. N-Acetylcysteine ethyl ester (NACET): A novel lipophilic cell-permeable cysteine derivative with an unusual pharmacokinetic feature and remarkable antioxidant potential. *Biochem Pharmacol* 84, 1522–1533 (2012).
10. Tosi, G. M. *et al.* Superior Properties of N-Acetylcysteine Ethyl Ester over N-Acetyl Cysteine to Prevent Retinal Pigment Epithelial Cells Oxidative Damage. *Int J Mol Sci* 22, 600 (2021).
11. Thevaranjan, N. *et al.* Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. *Cell Host Microbe* 21, 455-466 e4 (2017).
12. Annunziata, F. *et al.* Paneth Cells drive Intestinal Stem Cell Competition and Clonality in Aging and Calorie Restriction. *Eur J Cell Biol* 151282 (2022) doi:10.1016/j.ejcb.2022.151282.
13. Freter, R. *et al.* Establishment of a fluorescent reporter of RNA-polymerase II activity to identify dormant cells. *Nat Commun* 12, 3318–16 (2021).
14. Ermolaeva, M., Neri, F., Ori, A. & Rudolph, K. L. Cellular and epigenetic drivers of stem cell ageing. *Nature reviews. Molecular cell biology* 19, 594–610 (2018).
15. Neri, F. *et al.* Intragenic DNA methylation prevents spurious transcription initiation. *Nature* 543, 72–77 (2017).