*Consiglio di Dipartimento di Medicina Clinica e Sperimentale – riunione del 16.10.2019 – allegato 14/A*

# PROPOSTA PROGETTO DIPARTIMENTI DI ECCELLENZA

**TITOLO DEL PROGETTO:**

**Dysregulation of Circadian Rhythms in Parkinson’s Disease: a translational study from *in vitro* biochemical characterization of patient iPSC-derived neurons to *in vivo* evaluation of physical exercise.**

# DESCRIZIONE DELLE ATTIVITA’ PROGETTUALI

***Background and workplan***

Increasing evidences highlight a tight connection between circadian rhythms and metabolism with its dysregulation being increasingly recognized in many human pathologies. Mitochondria play a known pivotal role in cell bioenergetic and in addition function as a signaling hub controlling cell fate. Recent studies show that the mitochondrial quality control (fission/fusion mechanisms, mitophagy and mitochondria biogenesis) as well as mitochondrial respiration and oxidative phosphorylation activity are controlled by circadian rhythms. Parkinson's disease (PD) is a chronic, progressive neurodegenerative disease characterized by a selective loss of dopaminergic neurons of the

substantia nigra compacta. While most cases of PD occur sporadically involving unknown environmental factors or spontaneous mutations, several genetic mutations, were identified as responsible of early onset PD cases. This subset of monogenic PD cases has been associated with mutations in a set of *PARK* (*PARK1-18*) genes with many of these impinging in controlling the mitochondrial function. Although many studies have demonstrated the occurrence of circadian dysfunctions as well as mitochondrial dysfunction in PD, it is still to be defined whether this two factors act as an interplay in the onset and progression of PD. In the presented project we intend to investigate the correlation between the mitochondrial function and the cell-autonomous circadian clocks (i) in fibroblast from PD patients with PARK genes mutations and (ii) in dopaminergic neurons (DA) differentiated from induced pluripotent stem cells (iPSCs) derived from those fibroblasts. We will explore the signaling pathway possibly underlying alterations in the circadian clock-dependent mitochondrial function related to PD. The aim is to disentangle a hitherto unappreciated mechanism in the development of PD and conceivably in other neurodegenerative diseases. These findings will provide the mechanistic substrate by which chrono-therapy in PD can be developed. The basic study will be implemented with two applied-research investigations encompassing: i) the potential beneficial effect of nutraceutic compounds contained in extract of pomegranate juice and of metabolite precursors in recovering mitochondrial dysfunction in PD-derived cells (to be tested *in vitro*); ii) the impact of physical exercise in improving/re-establish circadian biological rhythms in PD patients (to be tested *in vivo*).

The workplan of the presented project is structured in several interconnected workpackages/tasks and will benefit of the multidisciplinary contributions of the teams’ components.

Task 1. Referring components: Proff. A. Bellomo and M. Altamura, Psychiatrists; Proff. P. Ricci, L.

Cipolloni, Medical Legals; Proff. T. Santantonio and S. Lo Caputo, Infectiologists.

Task 2. Referring components: Proff. N. Capitanio, C. Piccoli, R. Scrima, C. Pacelli and Dr. O. Cela,

Biochemists; Prof. C. Gallo, Computer Scientist.

Task 3. Referring components: Proff. C. Porro and T. Trotta, Anatomists

Task 4. Referring components: Proff. D. Colella, I. Sannicandro, Dr A. P. Di Tore and Dr S.

Bellantonio, Pedagogues in Sport Science; Prof. G. Guglielmi, Radiologist; Proff. P. Fiore and A. Santamato, Physiatrists.

***SPECIFIC PROJECT BREAKDOWN***

**Task1**: Recruitment of patients affected by early-onset familiar PD with established monogenic pathogenic mutations in the *PARK* genes family recovered in the clinics of the "Ospedali Riuniti" Hospital of Foggia. Their enrolment in the study will follow approval of the ethical committee and informed consent of the patients. These latter aspects will be handled by Prof. Ricci. Low-invasive skin punch biopsy will be carried out in selected patients and age-matched healthy subjects in order to isolate fibroblasts that in Tasks 2-3 will be used for the ahead described investigations.

**Tasks 2 - 3**: The fibroblasts obtained from PD patients and healthy subjects will be expanded in cultures and subjected to well-established protocols to synchronize the cell-autonomous clockwork machinery. The synchronization will be followed by an extensive biochemical and molecular characterization over a period of at least 48 h every 3 h after synchronization, in order to evaluate the circadian-clock dependent mitochondrial metabolism. The main investigations will be carried out to attain the aims described ahead.

**Aim 1**. **(i)** Metabolic fluxes analysis by the SeaHorse technology with direct measuring in live cells of the oxygen consumption rate (OCR) and simultaneously of the Extracellular acidification rate (ECAR). The Seahorse technology provides a standard and comprehensive method to assess the key parameters of mitochondrial OXPHOX and glycolytic activity, respectively (carried out by Pacelli and Scrima). **(ii)** Imaging study of the mitochondrial compartment to investigate features of the organelle dynamics/quality control and involvement in redox homeostasis, using specific fluorescent probes. In particular, high-resolution time-lapse confocal microscopy imaging will allow to acquire not only spatial information, but also temporal information (carried out by Piccoli). **(iii)** Gene/protein expression by Real Time PCR and western blotting of the major core-clock circadian oscillators (e.g. Bmal1, Clock, Period and Cryptochrome, NRD1) and of factors controlling mitochondrial dynamics (e.g. FIS, DRP1, PINK 1, Parkin) and biogenesis (sirtuins and PGC-1α) will be assessed (carried out by Scrima).

**Aim 2**. In a succeeding step, we will extend our evaluation carrying out the above mentioned analyses in iPSC (induced pluripotent stem cells)-derived neurons from human fibroblasts of patients

with PD-related gene mutations and age-matched controls. In order to obtain iPSC from fibroblasts, we will use a virus-free reprogramming technology consisting of nucleofection with episomal plasmids. This technique avoids both the risks of mutagenesis caused by the insertion of the virus in the human genome and the uncontrolled reactivation of the virus itself, making these cells safer even for future use in cell therapy (carried out by Capitanio and Pacelli).

The cells obtained will be evaluated for the acquired pluripotency by the ability to form teratomas when transplanted in immune-deficient mice (in collaboration with the "Stem Cell research group" of the "Mendel Instutute" in Rome). Additionally, stemness markers (Oct4, Sox2) will be assessed by immunocytochemistry followed by confocal imaging. Once confirmed the stemness status of the generated iPSCs, we will commit them to neuronal dopaminergic differentiation using well established protocols and commercially available kits (carried out by Pacelli). The successful differentiation will be evaluated by analyzing the expression of the thyroxine hydroxylase, a marker of dopaminergic neurons. We predict that in fibroblast cells as well as in iPS-derived DA neurons, PD- related gene mutations will lead, besides mitochondrial dysfunction, alterations in the clock gene expression. As proof of principle we will silence selected genes of interest in control and PD patients iPSC-derived DA neurons by RNA-interference methodologies (carried out by Pacelli, Scrima, Cela). These analyses might be of clinical relevance in the context of therapeutic strategies.

Furthermore, given the well-characterized property of mesenchymal stem cells (MSCs) to promote intercellular communication through microvescicles (MV) release, we will explore whether iPSCs are featured with the same competence. To this aim control and PD-patient-derived iPSCs both under non-syncronized and syncronized conditions will be assessed for the release of MV in the medium. The MV will be isolated by ultracentrifugation and characterized by flow-cytometry (performed by Porro and Trotta). In addition, the possibility that large MVs can function as cargos for mitochondria (as already demonstrated for MSCs) will be evaluated by using mitochondria-selective probes and analysis by flow-cytometry and confocal microscopy (carried out by Porro and Trotta).

**Aim 3**. Using pharmacological approaches, we will modulate the SIRT1-PGC-1α signaling pathway that proved to be involved in circadian clock-dependent mitochondrial function and in its dysfuntion. The cells (fibroblasts and iPSC-derived DA neurons from healthy and PD-patients) will be treated with nicotinamide riboside (NR), a natural NAD+ precursor, aiming at increase NAD levels in our cellular models, that will activate the SIRT1- PGC-1α axe (carried out by Scrima). In addition, the cells will be

treated with extract from pomegranate juice (in collaboration with the local Department of Agronomy) aiming at inducing sirtuin activity (carried out by Cela); further fractionation of the extract will be performed to identify the bioactive molecule(s). After each treatment, we will perform the same experiments described in Aim 1.

All the experimental data obtained from synchronized cells will be will be analyzed by "cosinor fitting" applying the following equation: f(t) = M + Acos(t2π/P + φ) + st; with M = mesor (Midline Estimating Statistic Of Rhythm); A = amplitude of oscillation, P = period, φ = acrophase (the phase of the maximum in relation to a fixed reference time), s = slope. The best fit will be attained using optimized algorithms to minimize the chi square and to compensate eventual rhythm-attenuation (developed by C. Gallo). This will allow to evaluate accurately differences between control and PD patients cells regarding circadian oscillation of the different biological processes analyzed.

The absence of occasional contaminations of the cultured cells (fibroblasts, iPSCs, iPSC- derived DA neurons) with microorganisms will be routinely assessed with specific attention to micobacteria because of their KCN-insensitive oxygen consumption activity which might interfere with measurements of the mitochondrial respiration (carried out by Proff. Santantonio and Lo Caputo).

**Task 4.** The selected patients from task 1 as well as a control group consisting in age- matched healthy subjects, will be enrolled in a personalized exercise training program (developed by Proff. Fiore and Santamato) following recommendation and data from the “Parkinson’s Outcome Projects” [[www.parkinson.org](http://www.parkinson.org/) › Parkinsons Outcomes Project Report to the Community]. The regimen of the physical therapy will be personalized depending on the clinical trait of the patient and will encompass: a) aerobic exercises (30 min 5 times a week); b) strengthening exercises (2-3 days a week) to combat posture and strength changes; c) flexibility/stretching exercises (at least 3-4 times a week); d) deep breathing (daily) (followed by Proff. Colella, Sannicandro, Di Tore and Bellantonio). The program will last at least six months and the outcome of the physical therapy in terms of motor control and cognitive performance will be clinically evaluated at three months and at the end of the program (evaluated by Proff. Fiore, Santamato, Bellomo). All the patients enrolled in the exercise training program will be subjected to functional Magnetic Resonance Imaging (fMRI) before the beginning of the training program and after six months from its initiation (carried out by Prof. Guglielmi)

.

Blood samples from PD patients and healthy subjects will be collected, following informed consent, before the beginning of the physical exercise program and at 3 and 6 months from its initiation. The blood samplings for each individual will be collected at consecutive intervals of 6 h for a total of 24 h (i.e. at 00.00, 06.00, 12.00, 18.00). The leukocyte fraction will be isolated from the whole blood and the mRNA extracted using well-established protocols. The expression profiles of the clock genes (BMAL1, CLOCK, PERs, CRYs, RORs, REV-ERBs) will be assesses by quantitative RT-PCR (carried out by Piccoli, Scrima, Pacelli and Cela).

# OBIETTIVI INTERMEDI E FINALI DEL PROGETTO

**Intermediate outcomes**

* Validation of the circadian clock-dependent mitochondrial function and dynamics in selected PD patient-derived and age matched controls fibroblasts.
* Validation of the circadian clock-dependent mitochondrial function and dynamics in selected PD patients and age-matched controls iPSCs-derived DA neurons.

# Final outcomes

* Unveiling the signaling pathway(s) and molecular determinants underlying the alterations in the circadian clock-dependent mitochondrial function in selected PD patient-derived and age- matched control fibroblasts and iPSCs-derived DA neurons and identification of new therapeutic targets.
* Assessing the effect of physical activity on circadian clock-dependent mitochondrial function in control an PD patients.

## INDICATORI PER LA VERIFICA DEL RAGGIUNGIMENTO DEGLI OBIETTIVI INTERMEDI E FINALI

* Number of publications of the obtained results in peer-reviewed journals indexed in PubMed (at least 2/year).
* Number of reports as oral or poster presentations in international congresses (at least 2/years).
* Training of non-structured young investigators involved in the project (under graduate students, PhD students).
* Level of success in the evaluation of proposed projects, related to the presented, for grant support.

## COMPONENTI DEL GRUPPO DI RICERCA

|  |  |  |  |
| --- | --- | --- | --- |
|  | Nome | Inquadramento universitario /  SSD | Ruolo/attività progettuale |
| 1 | Nazzareno Capitanio | PO / BIO10 | Task 2 |
| 2 | Claudia Piccoli | PO / BIO10 | Task 2 |
| 3 | Rosella Scrima | PA / BIO10 | Task 2 |
| 4 | Consiglia Pacelli | RTD-B / BIO10 | Task 2 |
| 5 | Antonello Bellomo | PA / MED25 | Task 1 |
| 6 | Chiara Porro | RU / BIO16 | Task 3 |
| 7 | Teresa Trotta | RU / BIO16 | Task 3 |
| 8 | Dario Colella | PO / M-EDF01 | Task 4 |
| 9 | Italo Sannicandro | RU / M-EDF01 | Task 4 |
| 10 | Pio Alfredo Di Tore | RTD / M-EDF01 | Task 4 |
| 11 | Sergio Bellantonio | RTD / M-EDF01 | Task 4 |
| 12 | Giuseppe Guglielmi | PA / MED36 | Task 4 |
| 13 | Pietro Fiore | PO / MED34 | Task 4 |
| 14 | Andrea Santamato | PA / MED34 | Task 4 |
| 15 | Teresa Santantonio | PO / MED17 | Task 1 / Task 2 |
| 16 | Sergio Locaputo | PA / MED17 | Task 1 / Task 2 |
| 17 | José Ramon Fiore | RU / MED/17 | Task1 / Task 2 |
| 18 | Luigi Cipolloni | PA / MED/43 | Task 1 |
| 19 | Crescenzo Gallo | RU / ING-INF05 | Task 2 |