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PRIN: PROGETTI DI RICERCA DI RILEVANTE INTERESSE NAZIONALE – Bando 2022
Prot. 20224CY2L9
PART A

1. Research project title

Pathogenic insights and search for biomarkers in RFC1-ataxia/CANVAS: a model to a deeper understanding of molecular mechanisms underlying late-onset neurodegeneration.

2. Duration (months)

24 months

3. Main ERC field

LS - Life Sciences

4. Possible other ERC field

5. ERC subfields

1. LS5_11 Neurological and neurodegenerative disorders
2. LS2_14 Genetic diseases
3. LS1_2 Biochemistry

6. Keywords

n°	**Testo inglese**
1.	RFC1
2.	CANVAS

nº	Testo inglese
3.	ataxia
4.	neuropathy
5.	mitochondria
6.	oxidative stress

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7. Principal Investigator

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8. List of the Research Units

nº	Associated Investigator	Qualification	University/ Research Institution	Registered office (address)	Operating office (address)	e-mail address
1.	SILVESTRI Gabriella	Ricercatore confermato	Università Cattolica del Sacro Cuore	Largo Gemelli, 1 - MILANO (MI)	City: Roma Address Largo F. Vito 1, 00168	gabriella.silvestri@unicatt.it
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9 - Substitute Principal Investigator (To be identified among one of the associated investigators participating in the project).

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10. Brief description of the proposal

CANVAS (Cerebellar Ataxia, Neuropathy, Vestibular Areflexia Syndrome), or RFC1-ataxia, is a recently characterized rare neurodegenerative disorder, due to a biallelic intronic AAGGG expansion in Replication Factor C subunit 1 (RFC1) gene. It is an adult-onset disorder, in which cerebellar ataxia, neuropathy and vestibular impairment variably combine. Data regarding natural history of RFC1-ataxia are missing, and the molecular mechanisms by which this dynamic mutation produces clinical manifestations are still unknown. Of note, recent literature data suggest that RFC1-ataxia is a common genetic etiology of adult-onset degenerative ataxia. This issue is particularly relevant, as its pathogenic characterization might also give insight on the mechamisms underlying more frequent neurodegenerative disorders also characterized by late onset , such as Parkinson's and Alzheimer disease.
Therefore, the aims of this study are i) to assess and compare sensitivity of different validated clinical rating scales in estimating global disease severity and/or progression of RFC1-ataxia, for future trial readiness; ii) to get insight about pathogenesis of RFC-1 ataxia by both human in vitro and in vivo studies, particularly concerning putative mechanisms related to defect in single-strand break repair (SSBR) and related mitochondrial function. The first objective will be addressed in detail on primary cultured fibroblasts derived by skin biopsies available from RFC1-ataxia patients and age-matched healthy controls, on which we will test the hypotheses of impairment of the DNA SSBR machinery and mitochondrial dysfunction as main pathogenic contributors. Patients' and controls' fibroblast cell lines will be also used to generate induced-Pluripotent Stem Cell-derived proprioceptive neurons, being a powerful model to verify the above proposed pathogenic model, together with data obtained on fibroblasts. Regarding in vivo studies, the occurrence of increased oxidative stress will be tested in blood samples from RFC1-ataxia patients and related controls by assessing an extensive panel regarding oxidative stress balance , together with Neurofilament light chain, also as potential biomarkers.

11. Total cost of the research project identified by items

Associated Investigator	**item A.1**	**item A.2.1**	**item B**	**item C**	**item D**	**item E**	**sub-total**	**Total**
SILVESTRI Gabriella	26.973	24.500	30.884	0	40.000	34.000	156.357	156.357
BARACCA Alessandra	25.144	24.000	29.486	0	12.000	55.000	145.630	145.630
Total	52.117	48.500	60.370	0	52.000	89.000	301.987	301.987

N.B. The Item B and TOTAL columns will be filled in automatically

- item A.1: enhancement of months/person of permanent employees
- item A.2.1: cost of contracts of non-employees, specifically to recruit
- item B: overhead (flat rate equal to 60% of the total personnel cost, A.1+A.2.1, for each research unit)
- item C: cost of equipment, tools and software products
- item D: cost of consulting and similar services
- item E: other operating costs

PART B

B.1

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1. State of the art

Inherited Cerebellar ataxias (CAs) are genetically heterogeneous neurodegenerative disorders mainly involving the cerebellum and its pathways. They are usually slowly progressive diseases, where CA can be also associated with other neurological or extraneurological signs (1). Among discoveries driven by recent advances in molecular genetics, particular mention deserves the Replication Factor subunit 1 (RFC1) gene, associated with an autosomal recessive (AR) CA named CANVAS (Cerebellar Ataxia, Neuropathy, Vestibular Areflexia Syndrome, OMIM #614575) (2). The related molecular defect is a biallelic microsatellite intronic RFC1 expansion, whence the terminology of RFC1-related ataxia (from now on RFC1-ataxia) (3).
Clinically, RFC1-ataxia is late-onset, slowly progressive, and growing evidence is showing that it may be the most common inherited adult-onset ataxia (2). Retrospective data on natural history of RFC1-ataxia would suggest that ganglionic proprioceptive neurons might be the first to be involved, followed by vestibular and then by cerebellar neurons (4).
In RFC1-ataxia, a pathogenic AAGGG pentanucleotide expansion replaces the wild-type sequence of 11 AAAAG repeats located in the poly(A) tail of an AluSx3 element in intron 2 of RFC1 (2). Such genomic region is highly polymorphic, and the RFC1 pathogenic expansions usually display more than 400 repeats in leukocytes (5). How this dynamic mutation leads to neurodegeneration is not understood. The recessive mode of inheritance may point toward a loss-of-function (LOF) mechanism, as it occurs in Friedreich's ataxia (FA), yet few preliminary studies indicated that the levels of both the RFC1 protein and its messenger RNA (mRNA) were unchanged in various patients' tissues (2). Moreover, no RNA foci, nor expanded antisense transcripts could be detected (2).
RFC1 encodes the largest subunit of replication factor C, a 5-subunit DNA polymerase accessory protein, that loads proliferating cell nuclear antigen (PCNA) onto DNA, and activates DNA polymerases delta and epsilon for synthesis of DNA strands during replication or its repair after damage (6). As neurons are cells with low replication rate, RFC1 might play a prominent role in DNA repair in central nervous system (CNS). However, no studies widely evaluated putative effects of the biallelic expansion on both function and interactions of RFC1 with other proteins involved in DNA replication and repair after damage.
Of note, other AR inherited ataxias, such as oculomotor apraxia (AOA) type 1, type 4 and type 5, and spinocerebellar ataxia with axonal neuropathy type 1 (SCAN1) share with RFC1-ataxia a similar clinical involvement (cerebellar plus peripheral nerve degeneration) (7). Indeed, all their related mutated genes code for proteins that take part to the DNA single-strand break repair (SSBR) machinery, which is a fundamental apparatus for DNA repair in CNS. SSBR is a 4-step process, including detection of single-strand breaks (SSBs), end-processing, gap-filling and ligation: proteins involved in end-processing include polynucleotide kinase phosphatase (PNKP), aprataxin (APTX), and tyrosyl-DNA phosphodiesterase 1 (TDP1), whose mutated genes cause AOA4, AOA1 and SCAN1, respectively (7). In gap-filling and DNA ligation, RFC1 is essential to recruit

polymerase delta, required to start DNA repair (6).
The SSBR pathway is also likely to be involved in mitochondrial DNA (mtDNA) repair, so disruption of RFC1 activity might also cause mitochondrial dysfunction in neurons. Of note, both FA and mitochondrial DNA polymerase gamma (POLG)-related ataxia, which recognize mitochondrial dysfunction as driving disease mechanism, also share with RFC1 ataxia cerebellar ataxia and sensory axonal neuronopathy as discriminative features (8).
Oxidative phosphorylation (OXPHOS) complexes' subunits are encoded by both mtDNA and nuclear genes. Therefore, alteration of function of RFC1 protein may accumulate mutations of either genome, eventually leading to defects of respiratory complexes and/or ATP synthase in mitochondria, that would induce deficiency of cellular energy, especially in neurons. Furthermore, OXPHOS impairment could increase generation of reactive oxygen species (ROS) (9), that may also be caused by impairment of the antioxidant defense systems of the cells and further predispose to mtDNA damage(10,11). Another important effect of ROS is the induction of permeability transition by opening the cyclosporine-sensitive pore (PTP) in the inner membrane that opens in response to elevated levels of Ca2+ ions in the mitochondrial matrix (12). In consequence, the mitochondria take in water, and their membranes rupture, disrupting the synthesis of ATP and ion homeostasis, eventually leading to cell death (13). Of note, PTP disruption has been described as a consequence of LOF variants in paraplegin gene, coding for a mitochondrial protein, causing SPG7, another form of degenerative ataxia plus spasticity (8)

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2. Detailed description of the project: methodologies, objectives and results that the project aims to achieve and its interest for the advancement of knowledge, as well as methods of dissemination of the results achieved

Our research proposal recognizes three primary aims: i) to assess and compare sensitivity of different validated clinical rating scales in estimating global disease severity and/or progression of RFC1-ataxia, ii) to assess potential circulating RFC1-ataxia biomarkers in a well characterized cohort of RFC1-ataxia patients vs controls, and iii) to establish patient-derived cell models to get insight about pathogenesis of RFC1-ataxia, particularly concerning putative molecular defects in SSBR and/or mitochondrial function.
The first two aims will be assessed in a longitudinal study cohort including 25 genetically determined RFC1- ataxia patients in follow-up at our Neurological Center , which operates at Fondazione Policlinico A. Gemelli IRCCS-Rome.
According to the involvement of cerebellar and proprioceptive systems in RFC1-ataxia, we have selected the following rating scales: Scale for the assessment and rating of ataxia (SARA) (14), International Cooperative Ataxia Rating Scale (ICARS) (14), sensory ataxia rating scale (SEARS) (15), Berg Balance Scale (BBS) (16), and Tinetti Assessment Tool (16). Vestibular symptoms will be assessed by Dizziness handicap inventory questionnaire (DHI) (17).
1) SARA is a clinical scale validated in degenerative ataxias, which assesses a range of different impairments related to cerebellar dysfunction. The scale is made up of 8 items related to gait, stance, sitting, speech, finger-chase test, nose-finger test, fast alternating movements and heel-shin test (14);
2) ICARS is another, recommended outcome scale for cerebellar ataxias, including 19 items and 4 subscales evaluating postural and gait disturbances, limb ataxia, dysarthria, and oculomotor disorders (14);
3) SEARS is a 10-item functional scale developed and validated to cover the clinical spectrum of manifestations in patients with sensory neuronopathies. It has been validated for any forms of sensory ataxia, including 10-meter walking test (10mWT) to assess gait speed. Scores range from 0 (no impairment) to 41 (maximal impairment) (15);
4) BBS for balance assessment is an ordinal scale ranging from 0 to 56, consisting of 13 items with

a score from 0 to 4 where 0 is the minimum performance and 4 is the maximum performance regarding the item required. This scale mainly evaluates the static balance and minimally the dynamic (16);
5) The Tinetti Assessment Tool is a simple test that measures both gait and balance abilities based on performing specific tasks. Scoring of the Tinetti Assessment Tool is done on a 3-point ordinal scale with a range of 0 to 2, where 0 represents the most impairment, while 2 represents independence. The individual scores are then combined to form three measures: an overall gait assessment score (maximum score 12), overall balance assessment score (maximum score 16), combined gait and balance score (maximum total score is 28). Scores below 19 indicate high risk for falls (16);
The DHI evaluates the self-perception of the incapacitating effects, on the quality of life, caused by dizziness. It comprises 25 items with a total score ranging between 0 and 100 points. DHI can be further subdivided into physical (DHI-P, 28 points), functional (DHI-F 36 points) and emotional (DHI-E 36 points) subscores. A higher score indicates a more severe handicap (17).
For statistical analysis regarding global clinical severity, at T0 (baseline) RFC1- patients will also undergo:
1) neurophysiological studies: EMG and sensory and motor nerve studies, motor and sensory evoked potentials recorded at the four limbs;
3) vestibular function assessment by video head-impulse-test.
The following data will be also collected: age at onset (AAO) of symptoms, age at enrollment (AAE), disease duration (DD); presence of unexplained cough and/or swallowing disturbances; brain and spinal cord MRI neuroimaging data. Statistical analysis will be performed by Pearson's correlation coefficient to compare global and item scores obtained by each scale also with DD, disability stage based on the SPATAX disability score (0: no functional handicap, 1: no functional handicap but signs at examination, 2: mild, able to run, unlimited walking, 3: moderate, unable to run, limited walking without aid, 4: severe, walking with one stick, 5: walking with two sticks, 6: unable to walk, requiring a wheelchair, 7: confined to a bed), and major diagnostic findings.

The second aim will be addressed by collecting blood samples from the RFC1- ataxia cohort (25 patients) and a comparable cohort of age-and sex matched healthy controls (no smokers and without apparent history of any neurological disorders) enrolled to this purpose for the assessment of an extensive panel of biomarkers of oxidative stress (OS) and Neurofilament light chain, now considered as a reference standard of neurodegeneration, at both T0 (baseline visit) and T1 (one-year follow-up visit).
Blood samples will be collected to obtain serum, plasma and RNA. For serum analyses, 9 cc of blood in Vacuette (CAT serum sep clot activator) tube will be drawn, centrifuged within 1 hour, aliquoted in multiple 50 uL aliquots, and stored at -80°C. For plasma analyses, 9 cc of blood will be drawn into EDTA tubes and, after 30' at room temperature, will be centrifuged and aliquoted in 50 uL aliquots and stored at -80°C. Lastly, venous blood will be drawn into one 10 ml BD Vacutainer tube with EDTA, and total RNA will be extracted from leukocytes and reverse-transcribed into cDNA, that will be stored at -80°C. All the samples collected will be stored, then, at the end of the collection period, will be shipped and examined simultaneously in duplicate to avoid freeze–thaw cycles, in collaboration with the Laboratory of Molecular Medicine of the Bambino Gesù Hospital, IRCCS (Dr.ssa Fiorella Piemonte).
The oxidative stress panel will include:
- expression of the transcription factor NRF2 (Nuclear factor erythroid 2-related factor 2) and its downstream genes, analyzed by Real Time-PCR. NRF2 regulates many cytoprotective pathways through the activation of antioxidant defenses, inhibition of inflammation, improvement of mitochondrial function, and maintenance of protein homeostasis. Of note, changes in this pathway have been associated to several neurodegenerative diseases associated with mitochondrial dysfunction, in particular FA (18);

- serum markers related to antioxidant response [Superoxide Dismutase 1/2, Glutathione peroxidase 4, 15-Lipoxygenase, reduced glutathione (GSH) and oxidized glutathione (GSSG)], quinones detoxification [NAD(P)H quinone oxidoreductase 1], inflammation (Heme oxygenase 1), and glutathione metabolism (gamma-Glutamylcysteine ligase, Glutathione reductase) will be measured in sera by spectrophoto-fluorimetric assays and enzyme-linked immunosorbent assay (ELISA) (19);
- levels of by-products derived from membrane lipid peroxidation (4-hydroxynonenal, 15-Hydroxyeicosatetraenoic acid). These products and Plasma Neurofilament Light Chain (NfL) levels will be both assessed by Ella automated immunoassay (19).

Results obtained in both RFC1 patients and controls will be statistically compared by Mann Whitney U-test (BM SPSS Statistics for Windows, Version 24.0. Armonk, NY) to test if any biomarkers would be significantly different in RFC1 patients, thus potentially representing a disease biomarker. In case of positive results, to assess the role as a disease severity marker, their levels will be correlated both with global and item scores of the most sensitive clinical rating scale among those tested in the cohort. Also, their levels at T0 and T1 will be compared, to confirm significant difference between patients and controls, and especially to evaluate their potential role as disease progression biomarker.

The other major objective is represented by the deep characterization of RFC1 patient-derived cell lines, starting from fibroblasts, regarding the occurrence of putative SSB DNA repair defect and/or mitochondrial dysfunction.

Previous studies conducted on CANVAS patients' tissues apparently showed normal protein and transcripts levels of RFC1 (2), however studies evaluating putative RFC1 function and its interaction to other proteins involved in both DNA replication and repair after damage have not been performed so far. To this purpose, we will analyze in parallel patients and controls-derived primary fibroblasts (PF) (from 8 distinct RFC1 patients and 8 matched healthy controls).

RFC1 protein and mRNA levels will be quantified through Western blot (WB) and quantitative (q)PCR, respectively on total protein and RNA extracted from cell lysate. To assess the interactomic network of RFC1: proteins from nuclei and cytoplasms will be extracted and analyzed by mass spectrometry to compare the proteomic pool in RFC1 patients and controls cell lines. After RFC1 immunoprecipitation with a specific antibody, immunoprecipitated proteins bound to RFC1 will be characterized by mass spectrometry. Finally, to test response to DNA damage, cell lines of RFC1 patients and controls will be irradiated with ultraviolet (UV) light to induce SSBs. The following markers of the SSBR pathway will be studied through WB and Immunofluorescence (IF): Poly ADP ribose polymerase 1 (PARP1), X-ray repair cross-complementing protein 1 (XRCC1), PCNA, gammaH2AX and RFC1 (7, 20). Given the link between efficient DNA repair and cell survival, a viability assay will be also performed for each cell line (MTT assay protocol, Sigma), to compare the viability rate between RFC1 patients and controls (21).

Studies on oxidative stress and mitochondrial function

Our preliminary data support that mitochondrial dysfunction might play a role in the pathogenesis of RFC1-ataxia, as the electron microscopy revealed the presence of clear mitochondrial abnormalities in RFC1 patients' derived fibroblasts (Figure 1).

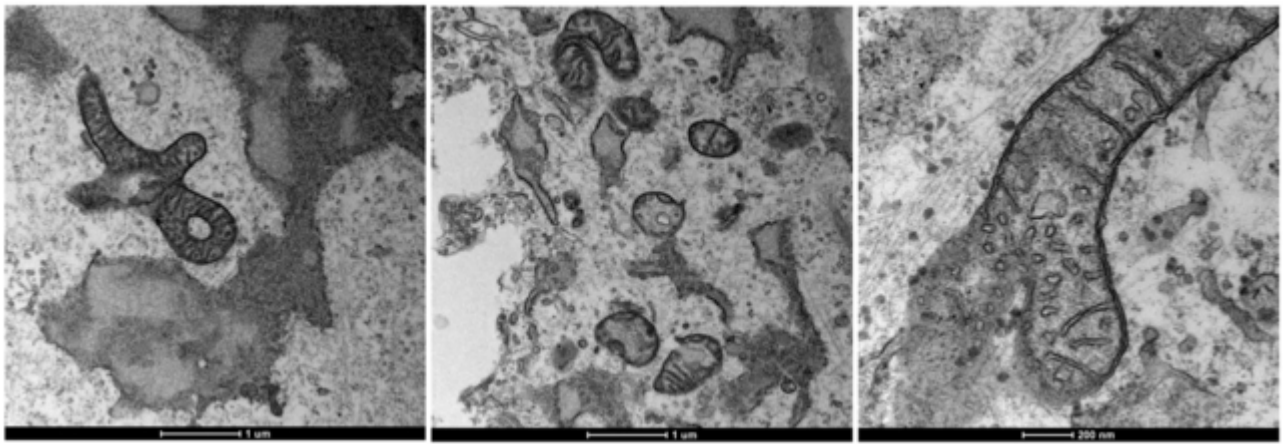

Fig. 1 Representative transmission electron microscopy images of one RFC1-ataxia patient fixed fibroblasts. Mitochondrial morphology is shown: swollen mitochondria, mitochondria characterized by low density and/or altered cristae, and donut (or ring-shaped) mitochondria. Scale bar, 1 μm and 200 μm.

To verify this hypothesis , we will extensively study mitochondria on RFC1 and controls fibroblasts by different approaches.

A) The presence of mitochondrial dysfunction is often associated with alterations either in dynamics, morphology and ultrastructure of mitochondria. These features will be investigated by confocal and transmission electron microscopy (TEM), as previously reported (12,22). For in vivo cell imaging, cells will be stained with Mitotracker Red, and Hoechst (nuclear probe). Images will be then captured by using a A1 confocal microscope, and image analysis will be performed using a specific software (Nikon Instruments). Cells will be scored into three categories: filamentous, intermediate, and fragmented mitochondria (22). Mitochondria ultrastructure will be analyzed on ultrathin fixed cells sections by a Tecnai G2 (FEI) TEM (12).

B) Mitochondrial dysfunction can also activate mitophagy (23,24), with consequent decrease of the mitochondrial mass contributing to cell energy defect. Mitochondrial mass will be assessed by immunoblot of porin and TOM20 (23,25), two mitochondrial proteins index of mitochondrial mass. As additional indexes, we will measure citrate synthase activity, a mitochondrial matrix enzyme, by spectrophotometry, and total mtDNA copy number by qPCR (13).

C) To highlight presence of impaired OXPHOS process in RFC1 cells, fibroblasts will be forced to utilize mitochondrial OXPHOS to synthesize ATP by substituting galactose to glucose in culturing medium. The entry of galactose into glycolysis occurs at a significantly lower rate than glucose (23,27). So, RFC1 and control fibroblasts will be cultured in DMEM containing either glucose or galactose as main energy substrate for up to 72 h, and cell growth will be analyzed (23). To deeply investigate OXPHOS, we will measure in digitonin-permeabilized cells (23) the oxygen consumption rate (OCR) under state 4, state 3 and uncoupled respiratory conditions, and the maximal rate of oligomycin-sensitive ATP synthesis. In addition, to definitely establish whether patients' fibroblasts are energy deficient, we will measure intracellular ATP levels (28). State 4 and State 3 respiration rates will be measured in the absence and in the presence of saturating ADP, respectively, and the uncoupled respiration in the presence of the uncoupler FCCP (Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone). The Complex I driven ATP synthesis rate will be measured under the state 3 respiratory conditions described above. The synthesized ATP or steady state ATP content of cells will be quantified by a luminometric method (30).

D) Mitochondrial dysfunction leads to mitochondrial membrane potential (MMP) changes and ROS production. Thus, MMP, a main functional parameter of mitochondria, will be estimated in cells by both fluorescence microscopy and flow cytometry to detect alterations of both the mitochondrial network and the inner mitochondrial membrane potential $\Delta\psi m$ (25,28). $\Delta\psi m$ will be measured by loading cells with tetramethylrhodamine methyl ester (TMRM), a lipophilic probe which enters

mitochondria in a Δψm-dependent manner. Cells will be incubated with TMRM either in the absence or presence of oligomycin, a specific inhibitor of the ATP synthase complex (31), and under FCCP. Multiple high-power fluorescence images of patients and controls fibroblasts will be acquired by fluorescence inverted microscope using IAS2000 software (Delta Sistemi, Roma, Italy). For quantitative Δψm analysis by flow cytometry, we will use a MUSE cell analyzer (Millipore, Billerica, MA, USA) applying the Flowing software (Centre for Biotechnology, University of Turku). Any evidence of mitochondrial alterations will be further addressed by immunoblotting of OXPHOS complexes (25).
E) In parallel to in vivo assessment, the possible contribution of higher ROS level and consequent cellular oxidative stress in the pathogenesis of RFC1-ataxia will be evaluated in cultured fibroblasts, by measuring anion superoxide radical ($O_2^{\bullet -}$) mitochondrial levels by the fluorescent probe MitoSOX Red (32), and cellular ROS level by the CellROX Orange fluorescent probe (33), using a MUSE cytometer. To explore whether ROS alterations depend on changes in the antioxidant defense systems of the cells, levels and activities of the main antioxidant enzymes and total and reduced glutathione level will be assessed by immunoblot and spectrophotometric assays, respectively (33,34).
F) Finally, the finding of significant mitochondrial alterations in RFC1 fibroblasts will prompt us to investigate their proneness to mitochondrial-induced cell death. Increased ROS induces permeability transition of the cyclosporine-sensitive pore in the inner mitochondrial membrane (12), which in turn causes mitochondria swelling and consequent disruption of both ATP synthesis and ion homeostasis, leading to cell death (13). Apoptosis activation will be assessed in patient and control cells in glucose enriched-DMEM medium at different times. Proneness to cell death will be evaluated by the Muse cell analyzer using the Muse Count and viability assay Kit, Annexin V and Dead Cell Assay Kit, and Caspase-3/7 Assay Kit.

Development of iPSCs-derived proprioceptive neurons
In the last few years, iPSCs have been used to generate models of human neurodegenerative diseases helpful to recapitulate the pathogenic process as it occurs in affected patients' brain. In this study, iPSCs will be differentiated into primary proprioceptive neurons, as sensory neurons result invariably affected in RFC1-ataxia patients. Three RFC1 and three controls PF culture lines will be induced into iPSCs and differentiated into neurons, as previously described (35). These cell lines will be deeply phenotyped, as described above for fibroblasts. Finally, development of this cell model will be also set for further research development on RFC1-ataxia.

Expected results
RFC1-ataxia is a slowly progressive neurodegenerative disease of recent genetic characterization, which variably encompasses distinct neuronal pathways involved in control of balance and motor coordination. Therefore, available clinical severity rating scales for degenerative ataxias should be also tested in RFC1-ataxia, in order to identify the most sensitive outcome tool among them, or if needed, derive a novel global rating scale specifically tailored for such genetic form, to be validated on larger RFC1 patients' cohorts. On the other hand, as for other slowly-progressive neurodegenerative disorders, clinical outcome tools may be not sensitive enough to detect disease changes in a relatively limited time-span, so accurate and easy accessible disease biomarkers could help to assess severity and possibly monitor evolution of neuronal damage over time. Both issues would be especially important for future trial readiness in RFC1 ataxia, as so far no data about the disease natural history by longitudinal studies are available.
The availability of a well characterized patients' cohort and of several cell lines obtained from RFC1 patients will allow to reach a deeper characterization of the pathogenic mechanisms underlying this relatively common disorder. The adult- onset and the clinical resemblance of this genetic ataxia with other neurodegenerative ataxic disorders recognizing a "mitochondrial" signature, and the putative role of the RFC1 protein in DNA repair, strongly support that a

mitochondrial dysfunction might be also at the basis of neurodegeneration in RFC1-ataxia. Moreover, such generated models and the obtained data will offer the chance in the next future of identifying and test potential therapeutic compounds able to rescue the aberrant phenotype in these preclinical RFC1 models.

Dissemination
Dissemination of the results to the scientific community will be achieved through contributions (oral presentations or posters) presented at National and International Scientific Meetings, and through writing scientific papers.
Research Unit 1 is a main clinical referee for degenerative ataxias, also including RFC1, allowing a constant communication with patients and their families. Dissemination of results regarding this research among the patients' community might be also achieved though active contacts established with Italian Ataxia patients Advocacy group (AISA). Based on the results of this proposal, the participation in international research networks (SPATAX) (Dr Silvestri, Dr Rossi) and the Young Investigators Ataxia Initiative (dr Rossi) will help to establish future collaborative research studies concerning RFC1-ataxia. The past fellowship of Dr S. Rossi (U1) at the UCL Neurogenetic Laboratory Queen Square Institute of Neurology, who worked on RFC1-ataxia under the supervision of Dr A. Cortese and Prof H. Houlden, both recognized leading researchers in this research field, started an ongoing collaboration that will offer intellectual exchange regarding the topic of this project.

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3. Project development, with identification of the role of each research unit, with regards to related modalities of integration and collaboration

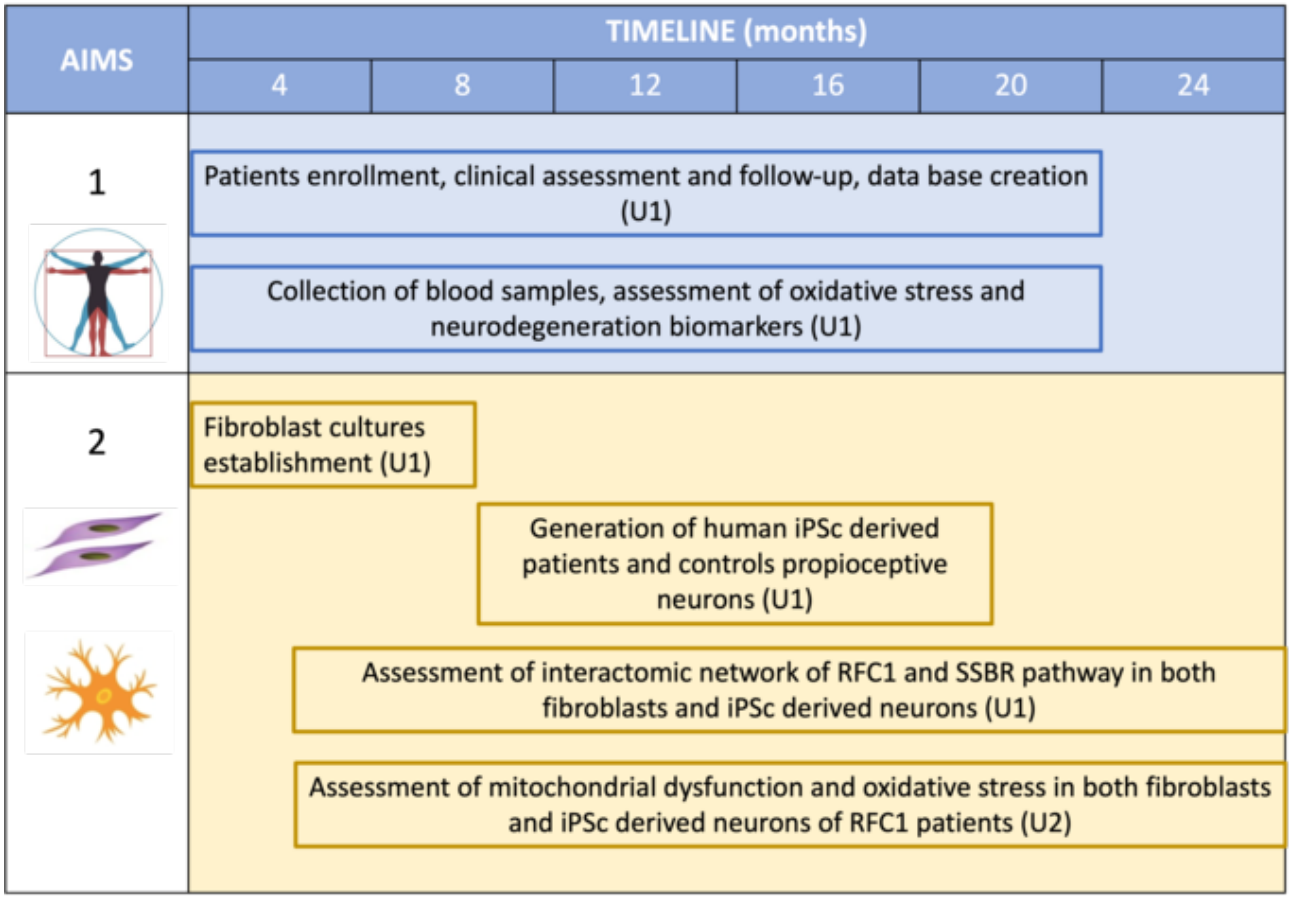
Research Unit 1 (U1): Competence and Qualification of the PI and the group The U1 is based at the Dept. of Neurosciences, Faculty of Medicine and Surgery, Università Cattolica del Sacro Cuore (UCSC) in Rome. Her leading researcher is Prof. Gabriella Silvestri (GS). GS, who has long and consolidated expertise on HSP and ataxia research, testified by her scientific production, leads a clinical research group on rare neurogenetic disorders, including HSPs and ataxias, collaborating with various National and International research groups on these topics (see CV). She promotes fellowships and PhDs of young neurologist researchers, as Dr. Salvatore Rossi, to improve their clinical and technical skills, favor their own scientific growth, and reinforce the whole research group activity. Indeed, DR Rossi spent six months at the UCL Neurogenetic Lab supervised by prof. Henry Houlden and dr Andrea Cortese, being involved in the research team focused on the molecular characterization of CANVAS. This expertise acquired during this fellowship, together with his past training during Residency in degenerative ataxias and other rare neurodegenerative disorders, having Dr Silvestri as tutor either in Clinics and in the Lab, allows Dr Rossi to handle both the clinical and the experimental part carried on at U1. Regarding the diagnostic assessment of the RFC1-ataxia cohort, prof Domenico Restuccia, an endorsed researcher in clinical neurophysiology, will supervise the protocol of the neurophysiological evaluation, and Prof Loredana Maggi, who has experience in neurorehabilitation, the clinical assessment of outcome scales. A research fellow will be also enrolled in the present proposal to accomplish the clinical-diagnostic assessment, data collection and analysis and biological samples management.
U1 operates at the Neurological Unit of the Policlinico Universitario A. Gemelli. Clinical resources include: 2 neurological wards able to admit 30 patients, a fully equipped neurophysiological lab, day-hospital and out-patient clinics for clinical and diagnostic activities, including a Tertiary Level Referral Center certified by Regione Lazio for diagnosis and follow-up of HSPs and degenerative ataxias, and lab technicians and facilities for related tissue samples processing, storage and establishment of primary fibroblasts cell cultures. Ten skin biopsies from RFC1 ataxia patients and 5 from age matched controls are currently stored at -20° and available for this proposal. Preliminary

data here presented regard one of these RFC1 biopsies.
U1 will carry on enrollment, clinical and diagnostic assessment, including rating scales and questionnaire administration, that will be carried out for each patient at T0 and T1 in two distinct sessions within the same week, and accomplish blood sample collection. Analysis of OS biomarkers and Nfl in blood samples will be carried out in collaboration with the Laboratory of Molecular Medicine of the Bambino Gesù Hospital, in the person of PhD Fiorella Piemonte, who is an expert in this field.
Definition of the interactomic network of RFC1, and response to DNA damage on PF from patients and controls will be also performed at U1. Moreover, differentiation of iPSCs into primary proprioceptive neurons will be performed thanks to our long-standing collaboration with dr.ssa Elisabetta Tabolacci (at the Institute of Genomic Medicine, Catholic University of Rome), who already published on this cellular approach, applied to different genetic conditions. A biologist (or biotechnologist) will be employed to manage these specific sections of the experimental plan.
Finally, U1 will coordinate the Research activities: GS will prepare a kick-off meeting, promote the creation of a data base for anonymized data entry, and periodically organize brief meetings to discuss the advancements protocol, exchanging results and other information related to project, and to devise new strategies, if needed.
Through an established collaboration with U2, U1 will provide fibroblasts and iPs derived neurons for extensive biochemical and molecular characterization concerning mitochondrial function
Research Unit 2 (U2) (Departimento di Scienze Biomediche e Neuromotorie, Università di Bologna)
U 2 - Competence and Qualification of the PI and the group: Prof. Alessandra Baracca (AB) and the research unit has a long-lasting experience in fundamental bioenergetics and a deep know-how in mitochondrial function and cellular energy metabolism in physiology and pathology, as detailed in the CV and publication list. The Unit contributed to unravel the pathogenic mechanisms of neurodegenerative diseases associated with either mitochondrial or nuclear genetic defects, showing the specific contribution of cellular bioenergetics and/or redox homeostasis impairments. Recently, the major interest of the RU was to shine light on energy metabolism and biochemical mechanisms leading to metabolic reprogramming of normal and transformed cells under conditions of reduced oxygen availability (hypoxia), in particular focusing on mitochondrial functions and dynamics.
Dr Valentina Giorgio, an Assistant Professor with a deep experience in cellular biochemistry, permeability transition pore (PTP) functional characterization, and PTP-dependent cell death, will help implementing the U2 experimental plan.
Established primary fibroblasts cultures derived from skin biopsies of 8 RFC1 patients and 8 age-matched controls obtained from U1 and iPSCs from three RFC1 and three controls PF culture lines will be investigated by U2.
U2 will analyze mitochondrial dysfunction and imbalance of ROS homeostasis in patient cells. In addition, U2 will investigate the proneness to apoptotic cell death of patients' cells. All the biochemical-molecular analyses will be carried out in both patient and age-matched control cells.
1) Dynamics, morphology and ultrastructure of mitochondria will be investigated in both RFC1-ataxia fibroblasts and iPSC-derived neurons by confocal and transmission electron microscopy
2) The bioenergetic phenotype of patients' cells will be investigated in depth as follows:
-Growth analysis of patient and control fibroblasts cultured in either glucose-enriched or glucose-free medium;
- Measurements of the respiration rate under state 3, state 4 and uncoupled respiratory conditions in patient permeabilized fibroblasts;
- Measurements of the oligomycin sensitive ATP synthesis rate (OXPHOS) in patient permeabilized fibroblasts;
- Evaluation of the cell energetic state by measuring the steady state ATP level and the ATP/ADP ratio in both patient fibroblasts and iPSC-derived neurons;
- OXPHOS complexes analysis by SDS-PAGE followed by immunodetection will be carried out in

both patient fibroblasts and iPSC-derived neurons;
- The possible contribution of diminished mitochondrial content to energy deficiency of patients' cells will be addressed quantifying level and enzymatic activity of proteins exclusively present in mitochondria (porin, TOM20, and citrate synthase). These data will be compared with those deriving from detection of mtDNA copy number carried out by U1;
- The mitochondrial membrane potential (Δψm), a bioenergetic parameter highly sensitive to OHPHOS dysfunctions and increased cellular level of ROS, will be measured by different methodological approaches.

3) To evaluate the relationships between mitochondrial function, redox homeostasis and cellular oxidative stress, the levels of ROS and main antioxidant defense systems will be investigated in fibroblast cell lines. Mitochondrial anion superoxide radical and cellular peroxide levels will be measured by using specific fluorescent probes. Also, total and reduced glutathione levels, and the levels and activity of main antioxidant enzymes, including superoxide dismutases, catalase, glutathione peroxidase and glutathione reductase will be determined.
4) The proneness to apoptotic cell death of patients' fibroblasts will also be investigated and compared with controls.
A full-time research fellow will be employed to address issue 3) and 4) of the experimental plan

The proposed timetable will be:



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4. Possible application potentialities and scientific and/or technological and/or social and/or economic impact

The results of this project might help to assess useful outcome tools and to shed light on the pathogenesis of this novel, and relatively common, form of inherited degenerative ataxia. Both

issues are in fact fundamental, preliminary steps in order to identify rational therapeutic approaches for RFC1-ataxia. In particular, the establishment and the deep molecular characterization of the pathogenic phenotype in distinct RFC1 patients derived cell models, especially iPSc-derived neurons, will hopefully allow also to select and test potentially effective drugs or other therapeutic compounds for this disabling disease. Also, this study aims to address another relevant information regarding future clinical trials for RFC1- ataxia, being the availability of objective outcome measures and possibly biomarkers, to assess progression in this slow neurodegenerative disorder: preliminary data obtained in a small but well characterized cohort could be informative to this scope.
Finally, increasing the knowledge about RFC1 pathogenesis might help to develop novel and effective therapeutic paths for other common late adult-onset neurodegenerative disorders, such as Parkinson's and Alzheimer disease. This issue might be particularly relevant in terms of Social Health Care, as increased life expectancy in Western countries is associated with a rise in the prevalence of age–related neurodegenerative disorders. A deeper understanding of the key mechanism(s) of neuronal apoptotic damage will aid to design comprehensive preventive/therapeutic strategies, in order to reduce this disease burden, which significantly impact particularly for our National Health System.

5. Financial aspects: costs and funding for each research unit

nº	**Total cost (euro)**	**Co-funding (item A.1) (euro)**	**MUR funding (other items) (euro)**
1.SILVESTRI Gabriella	156.357	26.973	129.384
2.BARACCA Alessandra	145.630	25.144	120.486
Total	301.987	52.117	249.870

6. Bibliography

1. Marsden JF. Cerebellar ataxia. Handb Clin Neurol. 2018;159:261-281.
2. Cortese A et al. Biallelic expansion of an intronic repeat in RFC1 is a common cause of late-onset ataxia. Nat Genet. 2019;51:649-658.
3. Cortese A et al. In: Adam MP et al., eds. GeneReviews®. Seattle (WA): University of Washington, Seattle; November 25, 2020.
4. Currò R et al. RFC1 expansions are a common cause of idiopathic sensory neuropathy. Brain. 2021;144:1542-1550.
5. Sullivan R et al. Cerebellar ataxia, neuropathy, vestibular areflexia syndrome: genetic and clinical insights. Curr Opin Neurol. 2021;34:556-564.
6. Majka J et al. The PCNA-RFC families of DNA clamps and clamp loaders. Prog Nucleic Acid Res Mol Biol. 2004;78:227-260. doi:10.1016/S0079-6603(04)78006-X
7. Caldecott KW. Single-strand break repair and genetic disease. Nat Rev Genet. 2008;9:619-631.
8. Dragašević-Mišković N et al. Autosomal recessive adult onset ataxia. J Neurol. 2022;269:504-533.
9. Nissanka N et al. Mitochondrial DNA damage and reactive oxygen species in neurodegenerative disease. 2018 FEBS Lett. 592:728-742.
10. Ray P.D. et al. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. 2012 Cell. Signal. 24:981–990.
11. Lenaz G et al. New insights into structure and function of mitochondria and their role in aging and disease. 2006 Antioxid Redox Signal. 8:417-37.

12. Galber C et al. The f subunit of human ATP synthase is essential for normal mitochondrial morphology and permeability transition. 2021 Cell Rep. 35:109111.
13. Bonora M et al. (2015) Oncogene 34: 1475-1486. Molecular mechanisms of cell death: central implication of ATP synthase in mitochondrial permeability transition
14. Perez-Lloret S et al. Assessment of Ataxia Rating Scales and Cerebellar Functional Tests: Critique and Recommendations. Mov Disord. 2021;36(2):283-297.
15. Martinez ARM et al. Sensory ataxia rating scale: Development and validation of a functional scale for patients with sensory neuronopathies. J Peripher Nerv Syst. 2019;24(3):242-246.
16. Martínez-Amat A et al. Effects of 12-week proprioception training program on postural stability, gait, and balance in older adults: a controlled clinical trial. J Strength Cond Res. 2013;27(8):2180-2188.
17. Jacobson GP et al. The development of the Dizziness Handicap Inventory. Arch Otolaryngol Head Neck Surg. 1990;116(4):424-427.
18. La Rosa P et al. The Nrf2 induction prevents ferroptosis in Friedreich's Ataxia. Redox Biol. 2021;38:101791.
19. Pastore A et al. Systemic Redox Biomarkers in Neurodegenerative Diseases. Curr Drug Metab. 2015;16(1):46-70.
20. Caldecott KW et al. The threat of programmed DNA damage to neuronal genome integrity and plasticity. Nat Genet. 2022;54(2):115-120.
21. van Meerloo J et al. Cell sensitivity assays: the MTT assay. Methods Mol Biol. 2011;731:237-245.
22. Del Dotto V. et al. (2017) OPA1 isoforms in the hierarchical organization of mitochondrial functions. Cell Rep., 19, 2557–2571.
23. Baracca A et al. Glucose plays a main role in human fibroblasts adaptation to hypoxia. 2013 Int J Biochem Cell Biol. 45:1356-65.
24. Malena A et al. Mitochondrial quality control: Cell-type-dependent responses to pathological mutant mitochondrial DNA. 2016 Autophagy. 12:2098-2112.
25. Sgarbi G et al. The role of the ATPase inhibitor factor 1 in cancer cells adaptation to hypoxia and anoxia. 2018 Biochim Biophys Acta Bioenerg. 1859:99-109.
26. Costanzini A et al. Mitochondrial Mass Assessment in a Selected Cell Line under Different Metabolic Conditions. 2019 Cells. 8:1454.
27. Bustamante E et al. High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase. Proceedings of the National Academy of Sciences of the United States of America 1977; 74:3735–9.
28. Barbato S et al. The inhibitor protein (IF1) of the F1F0-ATPase modulates human osteosarcoma cell bioenergetics. 2015 J Biol Chem. 290:6338-48.
29. Sgarbi G et al. Resveratrol preserves mitochondrial function in a human post-mitotic cell model. 2018 J Nutr Biochem. 62:9-17.
30. Sgarbi G et al. Human NARP mitochondrial mutation metabolism corrected with alpha-ketoglutarate/aspartate: a potential new therapy. 2009 Arch Neurol. 66:951-7.
31. Solaini G et al. The study of the pathogenic mechanism of mitochondrial diseases provides information on basic bioenergetics. 2008 Biochim Biophys Acta.1777:9415.
32. Sgarbi G et al. Hypoxia and IF_1 Expression Promote ROS Decrease in Cancer Cells. 2018 Cells. 7:64.
33. Sgarbi G et al. Hypoxia decreases ROS level in human fibroblasts. 2017 Int J Biochem Cell Biol. 88:133-144.
34. Baracca A et al. Biochemical phenotypes associated with the mitochondrial ATP6 gene mutations at nt8993. 2007 Biochim Biophys Acta. 1767:913-9.
35. Dionisi C et al. Primary proprioceptive neurons from human induced pluripotent stem cells: a cell model for afferent ataxias. Sci Rep. 2020;10:7752. Published 2020 May 8.

B.2

1. Scientific Curriculum of the Principal Investigator
Curriculum Vitae et studiorum: Gabriella Silvestri

Date of birth: 5/18/1963
Current Academic Rank: Assistant Professor in Neurology/ High Specialty Consultant in Neurology
Address: Fondazione Policlinico Universitario Agostino Gemelli,IRCCS
Università Cattolica del Sacro Cuore, Istituto di Neurologia, UOC Neurologia
L.go A.Gemelli 8, 00168 Roma, Italy
Work phone: +39 06/30154435
Fax: +39 06/35501909
E mail: gabriella.silvestri@unicatt.it
Scopus Author identifier: 35965821500
WOS researcher ID: J-8713-2018
ORCID ID 0000-0002-1950-1468

ACADEMIC EDUCATION AND TRAINING

7/1987: graduated in Medicine at the Faculty of Medicine of Catholic University of Sacred Heart Rome, Italy with honours
7/1991: - Board qualification in Neurology at the Department of Neurology, Faculty of Medicine of Catholic University of Sacred Heart Rome, Italy, with honours
-6/1991-1/1993: Postdoctoral research fellowship focused on molecular-genetic characterization of mitochondrial diseases under the supervision of Prof S. Di Mauro, Houston Merritt Center of Columbia University, New York, USA
- 10/1995: Phd in Neuroscience Faculty of Medicine of Catholic University of Sacred Heart Rome, Italy, with honours.

Academic and teaching profile
From 1/03/1997 and currently: Assistant Professor in Neurology - Faculty of Medicine and Surgery, Catholic University of Sacred Heart, Rome, Italy
National Scientific Certification Board as Associate Professor in Neurology Call year 2012 (DD n. 222/2012)

Since 2002:
-Teaching Courses in Clinical Neurogenetics, Postgraduate School in Neurology and Clinical neurophysiology, Faculty of Medicine Catholic University of Sacred Heart, Rome, Italy
-Teaching Courses in Neurology, School for Bachelor' Degree in Orthopaedic Techniques, Faculty of Medicine Catholic University of Sacred Heart, Rome, Italy
Since 2005:
- Teaching Courses in Neurology, Postgraduate School in Orthopaedics, Faculty of Medicine, Catholic University of Sacred Heart, Rome, Italy
Since 2016:
Teaching Courses in Neurology, Postgraduate School in Medical Genetics, Faculty of Medicine Catholic University of Sacred Heart, Rome, Italy

From 2003 to 2006:-Teaching Courses in Neurology, School for Bachelor' in Physical Rehabilitation, Faculty of Medicine, Catholic University of Sacred Heart, Rome, Italy

EMPLOYMENT

From 1/1/1991 to 2/15/1991 (part-time) - from 3/1/1994 to 8/15/1994 and from 1/11/94 and currently: Full-time Neurologist , current ranking of Alta Specializzazione (High Expertise), Fondazione Policlinico Universitario Agostino Gemelli,IRCCS UOC Neurologia

From 1993 to 2005: Consultant Neurologist at U.I.L.D.M.-Unione Italiana Lotta alla Distrofia Muscolare Sez. Laziale, (Italian Muscular Dystrophy Foundation, section of Latium)

RESEARCH PROFILE.
March 1991-January 1993: Postdoctoral fellowship, under the supervision of Prof. Salvatore DiMauro, Houston Merritt Research Center, Department of Neurology Columbia University on the clinical and molecular characterization of human mtDNA related disorders.
In 1999 P.I of a research project funded by Telethon Italy (project n 1227) focused on the definition of the pathogenic role of mitochondrial DNA duplications in human mitochondrial disorders associated with mtDNA deletions. (Final report sent to Telethon on 26/10/2000).
Starting form 2003 her clinical research activity has been more focused on myotonic dystrophy and on specific neurodegenerative disorders, including spinocerebellar ataxias and hereditary spastic paraplegias), being involved as main partner responsible of a research Unit in the following research proposals
- a multicenter research project on myotonic dystrophies funded by the Italian Ministry of University and Scientific research (M.I.U.R.) P.I. Dr. Fabrizio Loreni (Bando MIUR COFIN 2003).
- a multicenter research project funded by the Italian Ministry for Health (call RF-2003) on rare monogenic disorders associated with mental retardation , P.I. prof Corrado Romano
- two projects funded by ISS (bando Italia-USA malattie rare 2006), one focused on ataxic disorders , P.I prof N. Bresolin and the other one concerning the clinical and genetic characterization of hereditary spastic paraplegias P.I dr. FM Santorelli.
- two European multicenter studies on degenerative ataxias coordinated by Prof. Klockgether, University Hospital Bonn, Germany, named respectively RISCA (acronymous for Prospective study of individuals at risk for spinocerebellar ataxia type 1, type 2, type 3 and type 6 (SCA1, SC2, SCA3, SCA6) and SPORTAX (Sporadic degenerative ataxia with adult onset: Natural history study (SPORTAX-NHS)
- a multicenter research project funded by the Italian Ministry for Health (call RF-2010) for the creation of “The Italian Registry for Myotonic Distrophies” coordinated by prof. Giovanni Meola IRCSS San Donato Milanese
- EFFICACY OF METFORMIN ON MOTILITY AND STRENGTH IN MYOTONIC DYSTROPHY TYPE 1. A randomized, triple blind, placebo-controlled, multicenter clinical trial. funded by AIFA (Agenzia Italiana per il Farmaco Call 2016), Coordinating investigator prof. Roberto Massa (Univ. Tor Vergata Roma) Protocol AIFA EudraCT number: 2018-000692-32

-RILUZOLE (RILUTEK) IN PATIENTS WITH SPINOCEREBELLAR ATAXIA TYPE 7: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED PILOT TRIAL WITH A LEAD IN PHASE Coordinating Investigator: Giovanni Ristori MD, PhD, Codice AIFA-2016-02365063 N. EudraCT: 2018-000282-37

- Co-author of 157 papers published on peer-reviewed international ISI journals, 4 book chapters and more than 150 scientific meeting abstracts
Editor of a Special issue of Frontiers in Neurology 2020 “Myotonic Dystrophies: Developments in Research From Bench to Bedside”

Reviewer for international peer-reviewed journals (Muscle and Nerve, European Journal of Neurology, Neurology, Neurology Genetics, Journal of Neurology Neurosurgery and Psychiatry, Cognitive and behavioural Neurology, Journal of Pediatrics), for the AFM (French Muscular

Dystrophy Association)
Reviewer for the Italian Ministry for Education and Scientific Research M.I.U.R (VQR 2004-2010 ; 2011-2014,).
Editorial Board member for the Open Access "Brain Sciences
Review Editor for Neuromuscular Diseases Section of "Frontiers in Neurology"
Founder member of AIM (Italian Association of Myology)
Member of the Scientific Committee of aV.I.P.s (Associazione Italiana Vivere la Paraparesi Spastica) (Italian Association for the Hereditary Spastic Paraplegias)
Membro dell'Editorial Board delle riviste" e "Frontiers in Neurology, Section Neuromuscular Disorders.

Official H-index (Scopus): 35 WOS 33
Total Scopus citations: 4879 Total IF: 604
Total citations WOS Core collection: 4459, without self 4412

MEETING TEACHING/RELATORS ACTIVITIES

1) Docente Corso ECM "Advances in Fabry disease" 20/3/2014 presso Policlinico Universitario A.Gemelli, Roma Titolo "Discussion on neurological manifestations"
2) Docente Convegno con ECM Associazione AiVIPS (Associazione italiana Vivere la paraparesi spastica) Onlus in data 3/10/2015 presso Sala Convegni Casa Accoglienza Giovanni Paolo II , Roma
3) Corso ECM Regione Latium presso ASL Viterbo in data 16/4/2016 Malattie rare: atassia spinocerebellare tipo 2 (SCA2): dalla genetica alla presa in carico dei pazienti. Titolo" Atassia spino-cerebellare tipo 2 (SCA2): presa in carico assistenziale e riabilitazione"
4) Relazione per il XI Simposio di Genetica Clinica e Molecolare inerente " Genetica clinica e molecolare delle patologie cardiovascolari" tenutosi in data 23/6/2016
5) Docente per Corso ECM organizzato da SMO in data 28/10/2016 Malattie da espansioni di CAG: le atassie spinocerebellari e la DPRLA. Le atassie recessive. L'atassia di Friedreich.
6) relatore "Manifestazioni neurologiche nella malattia di Fabry in "Corso ECM Le malattie autoinfiammatorie e malattie lisosomiali nella medicina di precisione Roma 9-11 novembre 2017, UCSC , Fondazione Gemelli, Roma Titolo
7) Relatore "Il coinvolgimento neurologico in corso di malattia di Fabry. Meeting Cardionefrologia 2019 Titolo
8) Riunione Annuale Associazione AiVIPS (Associazione italiana Vivere la paraparesi spastica) Onlus presso "La Cascina Caratterizzazione clinica e genetica delle PSE: cosa ci ha insegnato e cosa ancora possiamo imparare? 2019

ADVISORY BOARDS
Invited member for one National (Milan, October 2021) and one international Advisory Board (webinar December 2021) "The management of alphamannosidosis in the adult population: clinical practice, current evidences and data gaps" organized by Chiesi S.p.a.

2. Scientific Curriculum of the associated investigators

1. **BARACCA Alessandra**
Curriculum Vitae

Name and Surname: Alessandra Baracca

Nationality: Italian
Date and Place of birth: 25 September 1957, Ravenna, Italy

Professional address: Department of Biomedical and Neuromotor Sciences
University of Bologna
Via Irnerio 48- 40126 Bologna.
Tel. +39-051-2091244,
Fax: +39-051-2091224
e-mail: alessandra.baracca@unibo.it

Qualification:

1982 Laurea cum laude in Biological Sciences, Faculty of of Mathematic, Physic and Natural Sciences - University of Bologna, Italy

1988 Ph.D. in Cellular Biology and Physiology, University of Bologna, Italy.

Position held and professional experience:

1983-1987 PhD student in Cellular Biology and Physiology, University of Bologna, Italy.

1988-91 Post-doctoral research fellowship supported by Consorzio Italiano Tecnologie Farmaci Invecchiamento (CITFI): Studies on cerebral ageing, Department of Biochemistry-University of Bologna.

1992-94 Postdoctoral fellow, Department of Biochemistry- University of Bologna, Italy.

1995-98 Research Contracts by Scuola Superiore di Studi Universitari e di Perfezionamento S. Anna- Pisa: Energy metabolism impairment in mitochondrial diseases.

1999-2015 Assistant Professor, Department of Biochemistry, Faculty of Medicine - University of Bologna, Italy.

2015-19 Professor, Associate of Biochemistry, Department of Biomedical and Neuromotor Sciences, School of Medicine and Surgery- University of Bologna, Italy.

2019-present Full Professor of Biochemistry, Department of Biomedical and Neuromotor Sciences, School of Medicine and Surgery- University of Bologna, Italy.

1986 Research fellow at the Department of Bioenergetics, Institute of Physiology, Czechoslovak Academy of Sciences, Prague (Dr J. Houstek).

2002 Guest fellow at the Department of Neurology, College of Physicians and Surgeons, Columbia University, New York Columbia University, collaboration with Prof. S. DiMauro and Prof. E Schon.

Main academic and scientific advisory duties: member of the Advisory board of the Dept of Biochemistry (a.a. 2001/02-2011/12), member of the Advisory board of the Dept of Biomedical and Neuromotor Sciences (2012/15; 2015/18), member of the Research Advisory board of the Dept of

Biomedical and Neuromotor Sciences (2018/21-2021/present), member of the Council of the School of Medicine and Surgery (2005/08; 2008/11; 2012/15; 2015/18), member of the Management Committee of the Italian Group of Bioenergetics and Biomembranes (GIBB) (2007/09 e 2009/2011).

Reviewer ad hoc for scientific journals, including: Biochemical Journal, Biochimica et Biophysica Acta, The International Journal of Biochemistry and Cell Biology, Neurobiology of desease, Clinical Science, International Journal of Developmental Neuroscience, Bioscence Reports, Cells, and Biochemical Society Transactions with expertise in mitochondrial pathophysiology and in structure, function and regulation of the ATP synthase complex.
Guest Editor of the Special Issue (2018) "Mitochondrial Bioenergetics in Cancer Cell Biology" in Cells.

Main teaching experience:
Lecturer of Biochemistry, Laurea Magistrale in Medicina e Chirurgia , School of Medicine and Surgery, University of Bologna, Italy (2004-present)

Lecturer of Biochemistry, Lauree triennali, School of Medicine and Surgery, University of Bologna, Italy:
-Tecniche della Prevenzione nell'Ambiente e nei Luoghi di Lavoro (2003-2017)
-Tecniche di Neurofisiopatologia (2016-present)
- Laurea triennale in Ostetricia (2017-present)

Lecturer of Biochemistry, Scuole di Specializzazione, School of Medicine and Surgery, University of Bologna, Italy
-Anatomia Patologica (2016-present)
-Patologia Clinica e Biochimica Clinica (2019-present)

Recent research interests
The scientific research covers a number of topics under the general heading of bioenergetics and in particular it focuses on aspects of mitochondrial pathophysiology. Specifically, the topics are:

-The pathogenic mechanisms of neurological disorders associated to mtDNA mutations as Neuropathy Ataxia and Retinitis Pigmentosa (NARP), Leigh Syndrome, and Leber Hereditary Optic Neuropathy (LHON).
- Hypoxia in mitochondrial physiology and pathology.
- Cellular bioenergetics and metabolism in normal and cancer cells
- The role of the endogenous inhibitor protein (IF1) of FoF1-ATPase ATP synthase) of mammalian mitochondria in normal and transformed cells.

Funded research projects (last 10 years)
PRIN 2010-11 Meccanismi mitocondriali della cancerogenesi (36 Mesi) - Coordinatore P. Bernardi, Univ. Padova. Componente U.R. di Bologna (G. Solaini)

Fondazione del Monte di Bologna e Ravenna (2014) -Macroarea: Innovazione tecnologica- (Prof. R. Bartesaghi) Titolo: Identificazione delle basi molecolari di patologie, quale strumento per la progettazione di terapie mirate- Linea 3 - Progetto di Ricerca: Metabolismo mitocondriale nei tumori: ruolo dell'inibitore endogeno dell'ATP sintasi nella cancerogenesi- Responsabile U.R.

Fondazione del Monte di Bologna e Ravenna (2015) Macroarea Tematica: Malattie oncologiche- Titolo: Modulazione Metabolica delle Cellule Tumorali in Ipossia. Responsabile Progetto di

Ricerca

Fondazione Cassa di Risparmio in Bologna (2018) Modulazione del metabolismo tumorale associata al silencing di IF1 come nuova strategia terapeutica. Responsabile Progetto di Ricerca

PRIN 2019-2022 (201789LFKB) The APP-mitochondria axis in iPSCs derived-neurones from Fragile X related-disorders (36 Mesi) - Coordinatore C. Bagni, Univ. di Torvergata, Roma. Componente U.R. di Bologna (V. Giorgio)

National and international research awards and recognition
1986-"Research Award" funded by the Italian Society of Biochemistry and Molecular Biology (SIB) as a means of supporting a research stay at the Department of Bioenergetics, Institute of Physiology, Czechoslovak Academy of Sciences, Prague.
1986-"Research Award" funded by the International Union of Biochemistry- International Union for Pure and Applied Bioenergetics Groups (IUB-IUPAB Bioenergetics Groups) as a means of supporting a research stay at the Department of Bioenergetics, Institute of Physiology, Czechoslova Academy of Sciences, Prague.

Top articles
-Literature-monitoring service provided by BioMedLib (May 2012)

List 1: Top 20 Articles, in the Domain of Article 17497220, Since its Publication (2007)
Evaluating mitochondrial membrane potential in cells. .SOLAINI G, SGARBI G, LENAZ G, BARACCA A: Biosci Rep; 2007 Jun; 27(1-3):11-21.
- ScienceDirect, Elsevier B.V.

Top 25 Hottest Articles
Biochemistry, Genetics and Molecular Biology > Biochimica et Biophysica Acta (BBA)-Bioenergetics
January to December 2011 and 2012 full year

Oxidative phosphorylation in cancer cells. SOLAINI G., SGARBI G., BARACCA A. (2011) BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1807, p. 534-542.

Hypoxia and mitochondrial oxidative metabolism. SOLAINI G., BARACCA A., LENAZ G., SGARBI G. (2010) BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1797, p. 1171-1177

-ScienceDirect, Elsevier B.V.

Top 25 Hottest Articles
Biochemistry, Genetics and Molecular Biology > Biochimica et Biophysica Acta (BBA)-Bioenergetics
January to March 2013 full year

Oxidative phosphorylation in cancer cells. SOLAINI G., SGARBI G., BARACCA A. C (2011) BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1807, p. 534-542.

-ScienceDirect, Elsevier 5 most Downloaded Articles
in Biochimica et Biophysica Acta (BBA)-Bioenergetics
January to July 2016.

Hypoxia and mitochondrial oxidative metabolism. SOLAINI G., BARACCA A., LENAZ G., SGARBI G. (2010) BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1797, p. 1171-1177.

-The most downloaded articles from ScienceDirect BBA-Bioenergetics in the last 90 days (2014, 2015, 2019):

Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F0 during ATP synthesis, by Baracca A. et al. (2003) BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1606, p. 137-146

Hypoxia and mitochondrial oxidative metabolism, by Solaini G. et al. (2010) BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1797, p. 1171-1177.

Editorial comments
-Comment by Prof. Hirano M. and Prof. DiMauro S: “Leber Hereditary Optic Neuropathy: Biochemical Lights in a Blurry Scenario in Arch Neurol (2005) 62, 711-12 to: Severe impairment of Complex I-driven adenosine triphosphate synthesis in Leber hereditary optic neuropathy cybrids, by Baracca et al ARCHIVES OF NEUROLOGY, vol. 62, p. 730-736

-Comment by Prof. Haller RG. and Prof. Vissing J.: “Drilling for Energy in Mitochondrial disease” in Arch Neurol (2009) 66, 931-32 to: Human NARP mitochondrial mutation metabolism corrected with alpha-ketoglutarate/aspartate: a potential new therapy, by Sgarbi et al ARCHIVES OF NEUROLOGY, vol. 66, p. 951-957.

-Editorial Comment by Sonia R. Singh, Jeffrey Robbins: “Desmin and Cardiac Disease An Unfolding Story” in Circ. Res. (2018) 122, 1324-1326 to: Desmin phosphorylation triggers preamyloid oligomers formation and myocyte dysfunction in acquired heart failure, by Rainer PP. et al. Circ Res. 2018, 122(10): e75–e83.

Bibliometric parameters of scientific production (Scopus):
IF (JCR 2020) = 424,123
Citations = 3511
H index = 28

The author presented 20 numbered scientific publications:
-in Sgarbi G et al. (2018) J. Nutr. Biochem.; and Barbato S. et al. (2015) J. Biol Chem., the author shares the senior authorship
-in Sgarbi G et al. (2006) Biochem. J., the author shares the first position
-in Malena A et al. (2016) Autophagy, the author is the corresponding author

3. Main Principal Investigator's scientific publications (Max. 20)

1. Galatolo D., De Michele G., Silvestri G., Leuzzi V., Casali C., Musumeci O., Antenora A., Astrea G., Barghigiani M., Battini R., Battisti C., Caputi C., Cioffi E., De Michele G., Dotti M. T., Fico T., Fiorillo C., Galosi S., Lieto M., Malandrini A.[...](2021). Ngs in hereditary ataxia: When rare becomes frequent. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES, vol. 22, p. 8490-N/A, ISSN: 1661-6596, doi: 10.3390/ijms22168490 - **Articolo in rivista**

2. Petrillo, Sara, Santoro, Massimo, La Rosa, Piergiorgio, Perna, Alessia, Gallo, Maria Giovanna, Bertini, Enrico Silvio, Silvestri, Gabriella, Piemonte, Fiorella (2021). Nuclear Factor Erythroid 2-Related Factor 2 Activation Might Mitigate Clinical Symptoms in Friedreich's Ataxia: Clues of an "Out-Brain Origin" of the Disease From a Family Study. FRONTIERS IN NEUROSCIENCE, vol. 15, p. N/A, ISSN: 1662-453X, doi: 10.3389/fnins.2021.638810 - **Articolo in rivista**
3. Riso V., Galatolo D., Barghigiani M., Galosi S., Tessa A., Ricca I., Rossi S., Caputi C., Cioffi E., Leuzzi V., Casali C., Santorelli F. M., Silvestri G. (2021). A next generation sequencing-based analysis of a large cohort of ataxic patients refines the clinical spectrum associated with spinocerebellar ataxia 21. EUROPEAN JOURNAL OF NEUROLOGY, vol. 28, p. 2784-2788, ISSN: 1351-5101, doi: 10.1111/ene.14868 - **Articolo in rivista**
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4. Main scientific publications of the associated investigators (Max. 20, for each research unit)

1. **BARACCA Alessandra**

1. Solaini G., Sgarbi G., Baracca A. (2021). The F1Fo-ATPase inhibitor, IF1, is a critical regulator of energy metabolism in cancer cells. BIOCHEMICAL SOCIETY TRANSACTIONS, vol. 49, p. 815-827, ISSN: 0300-5127, doi: 10.1042/BST20200742 - **Articolo in rivista**
2. Vilella R., Sgarbi G., Naponelli V., Savi M., Bocchi L., Liuzzi F., Righetti R., Quaini F., Frati C., Bettuzzi S., Solaini G., Stilli D., Rizzi F., Baracca A. (2020). Effects of

standardized green tea extract and its main component, EGCG, on mitochondrial function and contractile performance of healthy rat cardiomyocytes. NUTRIENTS, vol. 12, p. 1-20, ISSN: 2072-6643, doi: 10.3390/nu12102949 - **Articolo in rivista**

3. Anna Costanzini, Gianluca Sgarbi, Alessandra Maresca, Valentina Del Dotto, Giancarlo Solaini, Alessandra Baracca (2019). Mitochondrial Mass Assessment in a Selected Cell Line under Different Metabolic Conditions. CELLS, vol. 8, p. 1-14, ISSN: 2073-4409, doi: 10.3390/cells8111454 - **Articolo in rivista**
4. Gianluca Sgarbi, Giulia Gorini, Francesca Liuzzi, Giancarlo Solaini, Alessandra Baracca (2018). Hypoxia and IF1 Expression Promote ROS Decrease in Cancer Cells. CELLS, vol. 7, p. 1-12, ISSN: 2073-4409, doi: 10.3390/cells7070064 - **Articolo in rivista**
5. Sgarbi G, Barbato S, Costanzini A, Solaini G, Baracca A. (2018). The role of the ATPase inhibitor factor 1 (IF(1)) in cancer cells adaptation to hypoxia and anoxia. BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1859, p. 99-109, ISSN: 0005-2728, doi: 10.1016/j.bbabio.2017.10.007 - **Articolo in rivista**
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11. SOLAINI, GIANCARLO, SGARBI, GIANLUCA, BARACCA, ALESSANDRA (2011). Oxidative phosphorylation in cancer cells. BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1807, p. 534-542, ISSN: 0005-2728, doi: 10.1016/j.bbabio.2010.09.003 - **Articolo in rivista**
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15. SOLAINI, GIANCARLO, Harris DA, LENAZ, GIORGIO, SGARBI, GIANLUCA, BARACCA, ALESSANDRA (2008). The study of the pathogenic mechanism of mitochondrial diseases provides information on basic bioenergetics. BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1777, p. 941-945, ISSN: 0005-2728, doi: 10.1016/j.bbabio.2008.04.034 - **Articolo in rivista**
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17. Baracca A, Sgarbi G, Mattiazzi M, Casalena G, Pagnotta E, Valentino ML, Moggio M, Lenaz G, Carelli V, Solaini G. (2007). Biochemical phenotypes associated with the mitochondrial ATP6 gene mutations at nt8993. BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, vol. 1767, p. 913-919, ISSN: 0005-2728, doi: 10.1016/j.bbabio.2007.05.005 - **Articolo in rivista**
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5. Main staff involved (max 10 professors/researchers for each research unit, in addition to the PI or associated investigator), highlighting the time commitment expected

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List of the Research Units

Unit 1 - SILVESTRI Gabriella
Personnel of the research unit

nº	Surname Name	Qualification	University/ Research Institution	e-mail address	Months/person expected
1.	SILVESTRI Gabriella	Ricercatore confermato	Università Cattolica del Sacro Cuore	gabriella.silvestri@unicatt.it	6,0
2.	RESTUCCIA Domenico	Ricercatore confermato	Università Cattolica del Sacro Cuore	domenico.restuccia@unicatt.it	4,0
3.	MAGGI Loredana	Ricercatore confermato	Università Cattolica del Sacro Cuore	loredana.maggi@unicatt.it	6,0
4.	ROSSI Salvatore	Dottorando	Università Cattolica del Sacro Cuore	salvatorerossi309@gmail.com	6,0

Possible sub-unit

Surname	Name	Qualification	e-mail address	Months/person expected
PIEMONTE	FIORELLA	Phd	fiorella.piemonte@opbg.net	2,0
Institution: Laboratorio di medicina molecolare, IRCCS Ospedale Bambino Gesù, Roma				

Unit 2 - BARACCA Alessandra
Personnel of the research unit

nº	Surname Name	Qualification	University/ Research Institution	e-mail address	Months/person expected
1.	BARACCA Alessandra	Professore Ordinario (L. 240/10)	Università degli Studi di BOLOGNA	alessandra.baracca@unibo.it	4,0
2.	RIGHETTI Riccardo	Dottorando	Università degli Studi di BOLOGNA	riccardo.righetti3@unibo.it	6,0
3.	GRILLINI Silvia	Dottorando	Università degli Studi di BOLOGNA	silvia.grillini3@unibo.it	6,0
4.	ZANNA Claudia	Ricercatore a t.d. - t.pieno (art. 24 c.3-b L. 240/10)	Università degli Studi di BOLOGNA	CLAUDIA.ZANNA@UNIBO.IT	6,0

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6. Information on the new contracts for personnel to be specifically recruited

nº	Associated or principal investigator	Number of expected RTD contracts	Number of research grants expected	Number of PhD scholarships expected	Overall expected time commitment (months)
1.	SILVESTRI Gabriella	0	1	0	12
2.	BARACCA Alessandra	0	1	0	12
	Total	0	2	0	24

7. PI “Do No Significant Harm (DNSH)” declaration, in compliance with article n. 17, EU Regulation 852/2020. (upload PDF)

Piano attività di ricerca

Role of mitochondrial dysfunctions in the pathogenesis and progression of RFC1-ataxia/CANVAS

CANVAS (Cerebellar Ataxia, Neuropathy, Vestibular Areflexia Syndrome), or RFC1-ataxia, is an adult-onset rare neurodegenerative disorder (variable combination of cerebellar ataxia, neuropathy and vestibular impairment) due to a biallelic microsatellite intronic AAGGG expansion in Replication Factor C subunit 1 (RFC1) gene (1, 2). The molecular mechanisms by which this dynamic mutation produces clinical manifestations are still unknown.
RFC1 encodes the largest subunit of replication factor C, a 5-subunit DNA polymerase accessory protein, that loads proliferating cell nuclear antigen (PCNA) onto DNA, and activates DNA polymerases delta and epsilon for synthesis of DNA strands during replication or its repair after damage (3). RFC1 is involved in DNA single-strand break repair (SSBR), a 4-step process including detection of single-strand breaks (SSBs), end-processing, gap-filling and ligation. In gap-filling and DNA ligation, RFC1 is essential to recruit polymerase delta, required to start DNA repair (3).
The SSBR pathway is also likely to be involved in mitochondrial DNA (mtDNA) repair, so disruption of RFC1 activity might also cause mitochondrial dysfunction in neurons.

The aims of this study are:

i) to assess and compare sensitivity of different validated clinical rating scales in estimating global disease severity and/or progression of RFC1-ataxia,
ii) to assess potential circulating RFC1-ataxia biomarkers in a well characterized cohort of RFC1-ataxia patients vs controls,
iii) to establish patient-derived cell models to get insight about pathogenesis of RFC1-ataxia, particularly concerning putative molecular defects in SSBR and/or mitochondrial function.

The first two aims will be assessed in a longitudinal study cohort including 25 genetically determined RFC1- ataxia patients in follow-up at Neurological Center, Fondazione Policlinico A. Gemelli IRCCS-Rome.
The last aim is represented by the deep characterization of RFC1 patient-derived fibroblasts and iPSCs (induced-Pluripotent Stem Cell)-derived proprioceptive neurons regarding the occurrence of putative SSB DNA repair defect and/or mitochondrial dysfunction.

This part of the project foresees the activation of a post-doc fellowship to study the possible mitochondrial dysfunction involvement (analysis in parallel of primary fibroblasts derived from 8 distinct RFC1 patients and 8 matched healthy controls) with the following approaches:

- evaluation of mitochondrial network morphology alterations in live cells by confocal microscopy (Nikon Instruments) (4) and of mitochondrial ultrastructure organization in ultrathin fixed cells sections by transmission electron microscopy (Tecnai G2, FEI, TEM) (5);

- evaluation of mitochondrial mass decrease as a marker of mitophagy activation. Mitochondrial mass levels will be assessed by a) immunoblot of porin and TOM20 (two mitochondrial proteins index of mitochondrial mass) (6, 7); b) citrate synthase activity (a mitochondrial matrix enzyme) by spectrophotometry (8); c) total mtDNA copy number by qPCR (8);

- evaluation of impaired OXPHOS process by a) cell growth in galactose medium, a well-established condition to slow down the glycolysis and force the cells to rely on OXPHOS to

produce ATP (6); b) oxygen consumption rate (OCR) under state 4, state 3 and uncoupled respiratory conditions, and the maximal rate of oligomycin-sensitive ATP synthesis in digitonin-permeabilized cells (6); c) the complex I synthesized ATP or steady state ATP content of cells quantified by a luminometric method (9);

- evaluation of mitochondrial membrane potential (MMP, a main functional parameter of mitochondria) changes of cells loaded with tetramethylrhodamine methyl ester (TMRM, a lipophilic probe which enters mitochondria in a $\Delta\psi$m-dependent manner) by flow cytometry (MUSE cell analyzer, Millipore) (9, 10);

- evaluation of ROS production by measuring anion superoxide radical ($O2 \bullet -$) mitochondrial levels with MitoSOX Red fluorescent probe (11) and cellular ROS level with CellROX Orange fluorescent probe (12), using a MUSE cytometer. Antioxidant proteins levels, activities of the main antioxidant enzymes and reduced glutathione level will be assessed by immunoblot and spectrophotometric assays, respectively (12, 13).

- evaluation of mitochondrial-induced apoptosis by the Muse cell analyzer using the Muse Count and viability assay Kit, Annexin V and Dead Cell Assay Kit, and Caspase-3/7 Assay Kit (5, 8).

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3. Majka J et al. The PCNA-RFC families of DNA clamps and clamp loaders. Prog Nucleic Acid Res Mol Biol. (2004) 78:227-260.
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