CONVOCATORIA DE PROYECTOS DE INVESTIGACIÓN EN NEUROCIENCIA-2014

Fundación Tatiana Pérez de Guzmán el Bueno

TÍTULO: **HEREDOATAXIAS: ESTUDIO DE UN MODELO PATOGENICO COMUN MEDIANTE ANALISIS DE FUNCION MITOCONDRIAL**

TITLE: **HEREDOATAXIAS: ANALYSIS OF MITOCHODRIAL DAMAGE AS AN UNITARY PATHOGENIC MODEL**

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_____ _____

A. Jiménez Escrig J.I. Flores Nicolás

• Resumen del proyecto en español y en inglés

HEREDOATAXIAS: ESTUDIO DE UN MODELO PATOGENICO COMUN MEDIANTE ANALISIS DE FUNCION MITOCONDRIAL

Fundamentos: Las hererdoataxias están constituidas por un alto número de enfermedades que difieren en el desorden genético que las origina aunque cursan con síntomas clínicos relacionados. En la mayoría de ellas, la alteración causante de la enfermedad determina una disfunción mitocondrial que afecta la homeostasis intracelular.
Objetivos: 1) Delimitar la alteración genética responsable de la ataxia en cada paciente. 2) Valorar las consecuencias de las diferentes alteraciones genéticas sobre la función mitocondrial en cada uno de los pacientes atáxicos estudiados.
Metodología: Se incluirán 48 pacientes con heredoataxias en los que se hará una evaluación genética exhaustiva mediante estudio de los 88 genes causantes de heredoataxias, con tecnología HaloPlex y secuenciación masiva de ADN mitocondrial en PGM-IonTorrent. Posteriormente en estos pacientes se evaluarán las alteraciones mitocondriales (mitocondrias funcionales, mitocondrias totales, mitocondrias generadoras de ROS, mitofagia) y las señales indirectas de daño mitocondrial (formación de inflamasomas y actividad de caspasa-1).
Conclusión: Es un proyecto translacional en 2 fases, la primera traslada el desarrollo de las nuevas técnicas de secuenciación genómica a un grupo de pacientes necesitado de un estudio genético más amplio y la segunda fase valora los efectos de los genes mutados en una vía única que permita su uso como biomarcadores de severidad clínica y respuesta terapéutica.

HEREDOATAXIAS: ANALYSIS OF MITOCHODRIAL DAMAGE AS AN UNITARY PATHOGENIC MODEL

Abstract
Background: Heredoataxias are a group of different diseases caused by a number of gene mutations manifested by similar clinical symptoms. In most of them, the genetic disorder causes mitochondrial dysfunction that affects intracellular homeostasis.
Objectives: 1) To define the genetic causal mutation in a group of patients with heredoataxia and 2) to examine the mitochondrial dysfunction that cause the different mutations.
Methods: We plan to include 48 patients with heredoataxias that will undergo a thorough genetic evaluation by testing the 88 genes that are involved in heredoataxias with HaloPlex and mitochondrial DNA by next generation sequencing in a PGM-IonTorrent machine. Afterwards, we will examine in these patients mitochondrial disorders (functional mitochondria, total mitochondria and ROS generating mitochondria), and the indirect signs of mitochondrial damage (formation of inflamosomes and caspase-1 activity).
Conclusion: This is a translational project in 2 phases: the first one transfers the development of new genome sequencing techniques to a group of patients that need a broader genetic study and the second one studies the effects of the mutated genes in a common pathway that allow its use as biomarkers of severity and therapy response.

• Subvención solicitada a la Fundación especificando los montos para cada concepto financiable (No deben consignarse gastos indirectos)

-DNA extraction cost (10 euros x 48 cases) .....€ 480

- Massive sequencing genes in dominant and recessive ataxias:
(€ 510 x 48 cases) .....€ 24.480

The entire draft sequencing of candidate genes includes generation of a 48 sample library, the generation of 5 templates and one 318 Sequencing Chip price:

- Preparation of 48 Libraries = € 13,790, € 287.3 / sample.
- Preparing Templates = € 2620, € 54.6 / sample
- Preparation of sequencing: € 8,100; € 168.8 / sample (Total € 510.6 / sample).

- Cost of NGS of mitochondrial DNA: (186 € x 48 cases) .....€ 8.928

The entire mitochondrial DNA sequencing project, including the generation of 48 libraries, generation of 2 Sequencing Templates and 4 Chips 316:
- Preparation of 48 Libraries = € 5360, € 111.7 / sample.
- Preparing Templates = € 1128, 23.5 € / sample
- Preparation of sequencing 2440 €, € 50.8 / total sample (€186 / sample).

-material and means for cultures.....€ 4.000
-Reagents and labware .....€ 7.000
-Antibodies .....€ 8.000
-Kits valuation of mitochondrial damage, apoptosis and glutathione.....€ 8.000
-HPLC-Columns .....€ 2.000
-Overheads (publication, hardware, office, hiring of services, etc.).....€ 6.000
- Registration, and travel to a specialty congress 2nd and 3rd year.....€ 5.000

TOTAL project cost **€73.888**

• Financiación actual del grupo (IP y, en su caso, otros investigadores), especificando título del proyecto y monto de la subvención.

Ninguna en la actualidad.

• Financiación solicitada a otros organismos pendiente de resolución, especificando título del proyecto y monto de la subvención.

Título del proyecto: Desarrollo de una plataforma de diagnóstico de las ataxias genéticas mediante secuenciación de nueva generación
Monto de la Subvención: 50.472,00€
Organismo: Federación de Ataxias de España (FEDAES)

MEMORIA CIENTÍFICA

- Antecedentes, estado actual del tema y planteamiento del proyecto (máximo 3 páginas)

Ataxia derives from the Greek word *taxis* 'order' in our language, so literally means 'messy' and is a clinical symptom. For lack of knowledge about the disorder, the term ataxia is used as a synonymous in different diseases in which impairment in motor coordination is the main symptom. In light of current knowledge about this disorder, ataxia cannot be considered a single disease but a disorder that appears in a large number of different diseases, approximately 88. This means that, in clinical practice, it is not correct to say that a particular patient "has an ataxia" as a disease, since ataxia is not a single disease. Thus, two patients with ataxia may have very different diseases i.e. a mitochondrial lesion in Friedreich's ataxia or a channelopathy in SCA6. It is therefore necessary to know the specific type of ataxia that has a patient. Therefore, the first step to assess any treatment, prognosis, etc for every patient with ataxia is to find the underlying genetic defect that causes it, something usually not done in a high percentage of patients with ataxia.

Ataxias are considered to have a prevalence of 60 cases per 100,000 inhabitants (ref. 1-2). Thus, in a hospital with an area of 500,000 people 300 cases are expected, although this figure can be increased by frequent pilgrimage diagnosis of these patients. Ataxias can be classified as:

Nongenetic ataxias (infectious, demyelinating, toxic, metabolic, vascular, tumor, inflammatory and degenerative paraneoplastic), and genetic ataxias that can be recessive, dominant and mitochondrial

According to recent reviews (ref. 3-6), there are 52 recessive genetic ataxias (64 genes reported) (Table 1 of Annexe) and at least 41 dominant genetic ataxias (24 genes reported) (Table 2 of Annexe). Therefore, when a patient asks if there is any treatment for its ataxia, the answer has to be, "when we know what type of ataxia you have we will be able to answer your question".

The clinical approach to these patients is to exclude acquired causes of ataxia by physical examination, basic laboratory tests and immunology and the study with brain MRI and neurophysiological tests. Once acquired forms have been ruled out, or if the medical history suggests a genetic ataxia begins a genetic study to determine which the causative genetic mutation is among all causes listed in Tables 1 and 2.

Until recently it was not possible to undergo a complete genetic study of ataxia because the costs and time for studying a large number of genes made it unfeasible in clinical practice. Therefore, the genetic study was limited to examining the most common gene (frataxin, SCA1, 2, 3, 6, 7) which leads to more than 80% of cases with negative genetic studies (9-12) .

Recently this has changed since the development of techniques for next-generation sequencing (NGS) that allow to sequence a high number of genes, even an entire genome at low cost and in a short time (see ref.13-14). Therefore, in this project we intend to set-up this technique to the study of patients with ataxia for a rapid and efficient genetic ataxia type.

Molecular Pathogenesis of genetic ataxias: mitochondrial function as the unitary model

Cerebellar ataxias are caused by dysfunction of different pathways. The main routes are alterations in protein homeostasis, ion channel disorders, defects in DNA repair mechanisms and mitochondrial dysfunction. Given the evidence of mitochondrial involvement poliglutamine disorders, channelopathies, impaired DNA repair, etc. .., mitochondrial function will be primary or secondary affected in most of the hereditary ataxias. Mitochondria are cellular organelles responsible for energy supply to cells, essential for the development of its activity. Although the main function of mitochondria is to synthesize ATP via oxidative phosphorylation, they also regulate Ca2 + homeostasis, heme biogenesis and formation of metalloproteins containing iron-sulfur clusters. In addition to these metabolic roles, nowadays it is considered that mitochondria acts as potent damage sensor platforms for intracellular regulatory and effector signals in different biological processes such as apoptosis, necrosis and autophagy. In recent years has also been described that mitochondria coordinate the activation of innate immunity at different levels (15, 16). Currently, innate immunity is seen as an evolutionary conserved defense system in which an encoded receiver network identifies germline tissue changes and initiates an

immune response to regulate tissue homeostasis. These receptors are not exclusive innate immune cells but they also are expressed in somatic cells and each cell line expresses its own pattern of receptors.

Alterations in the integrity of the mitochondrial outer membrane are crucial for the release of cytochrome C and the formation of apoptosomas, protein structures that initiate the activation of caspase-9. This protease is implicated in the onset of programmed cell death (apoptosis), a key process in tissue homeostasis (17). It has been recently described that mitochondria are the integrator of different signs of damage and transforms this information in the generation of reactive oxygen species (ROS) and other signals that are detected by intracellular receptors innate immunity activating inflamasomas (18,19). Inflammasomes are multiproteic structures similar to apoptosomas involved in the activation of another protease, caspase-1, an enzyme that processes precursors of IL-1beta and IL-18 to its biologically active form (20). Excessive generation of ROS may be a determinant of cell death by necrosis. Through autophagy, cell engulfs damaged mitochondria (mitophagy) limiting the excessive production of free radicals (ROS), and limiting intracellular damage. Thus, mitophagy processes downregulate NLRP3 inflammasome activation (16, 18). Although initially inflammasomes structures were described as related to infection and inflammation, various studies implicate caspase-1 in the processing of more than 50 different proteins. It is considered that NLRP3 inflamasomas are involved in regulation of glycolysis and lipogenesis, so could be regarded as regulatory structures of intracellular homeostasis.

According to the above mentioned data the mitochondria seems to be a key regulator to the decision to induce cell death or to initiate repairing mechanisms depending on the cell damage. A high number of genetic ataxias are due to mutations that directly or indirectly disturb mitochondrial function (see Tables 1 and 2). Therefore, we hypothesize that these pathologies modify mitochondrial signals that could give rise to situations of excessive apoptosis, necrosis, affect the mitophagy rate or causes defects in immune intracellular repair mechanisms. These events in turn are dependent on other factors, notably metabolic and immune status of patients that could lead to different degrees of deleterious effect in diverse individuals affected by the same mutation and this would determine the severity of the pathology. Therefore we believe that an assessment of the rate of mitochondrial damage associating ROS generation with NLRP3/caspasa-1 and mitophagy activity as well as assessment of cell death may have a prognostic value helping the selection of the most appropriate therapy for patients with ataxia.

NLRP3 inflammasome activation is considered an underlying event in different metabolic diseases such as arteriosclerosis, type 2 diabetes, and metabolic syndrome. The control of such activity is now a therapeutic target in these diseases, some of them in advanced clinical trial stage (21). Interestingly, some of the metabolic diseases associated with NLRP3 inflammasome also are present in different types of ataxias. Assessing the activation rate of inflamasomas could allow the selection of patients that could benefit from therapies derived of trials of caspase-1 activity regulators that are being developed at present for the treatment of pathologies mediated by the activity dysfunction of this enzyme.

Table 1: Recessive ataxias

Disease	Gene/Protein
Abetalipoproteinaemia	MTP /Microsomal triglyceride transfer protein
Adrenomyeloneuropathy	ABCD1
AFG3L2-associated spatic-ataxia.neuropathy syndrome (SPAX5)	AFG3L2
Ataxia telangiectasia	ATM /Ataxia telangiectasia-mutated
Ataxia telangiectasia-like disorder	MRE11/Meiotic recombination 11
Ataxia with oculomotor apraxia, type 1	APTX /Aprataxin
Ataxia with oculomotor apraxia, type 2	SETX /Senataxin
Ataxia with vitamin E deficiency	TTPA /α-tocopherol transfer protein
Autosomal recessive ataxia of Charlevoix Saguenay	SACS/ Sacsin
Autosomal recessive cerebellar ataxia type 1	SYNE1/spectrin repeats-nuclear envelope 1
Autosomal recessive cerebellar ataxia type 2	ADCK3 (CABC1)/aarf-domain containing kinase 3
CAMOS (also SCAR5)	ZNF592
CARASIL	HTRA1
Cayman ataxia	ATCAY / Caytaxin

Cerebral folate deficiency	FOLR1
Cerebrotendinous xanthomatosis	CYP27/ Sterol 27-hydroxylase
Coenzyme Q10 deficiency	COQ2,PDSS1,PDSS2, COQ8 (ADCK3), COQ9 gene,COQ6
Congenital disorder of glycosilation type 1A	PMM2/phospho-manno-mutase
DNA polymerase γ disorders (mitochondrial recessive ataxia syndrome)	POLG- POLG2 / DNA polymerase γ
Fatty acid hydroxylase-associated neurodegeneration	FA2H(SPG35)
Female carriers of EIEE1	ARX
Friedreich's ataxia	FRDA/ Frataxin
Gaucher disease type III	GBA
Glutaric acidemia II	ETFA, ETFB, ETFDH
Infantile-onset spino cerebellar ataxia	C10orf2 /Twinkle, twinky
Krabbe disease	GALC
Late-onset Tay-Sachs disease	HEXA /hexosaminidase A
LBSL	DARS2
Marinesco-Sjögren's syndrome	SIL1 /BiP-associated protein
Megaloencephalic leukoencephalopathy with subcortical cysts	MLC1
Metachromatic leukodystrophy	ARSA
Niemann-Pick type C disease	NPC1, NPC2
Nonketotic hyperglycinemia	AMT, GLDC, GCSH
Optic atrophy deafness, ophthalmoplegia, myopathy, ataxia, neuropathy	OPA1
peroxisomal biogenesis factor 7	PEX7/ Peroxin 7
PHARC	ABHD12
Refsum's disease	PHYH/ Phytanoyl-CoA hydroxylase
Sandhoff's disease	HEXB /hexosaminidase B
Spastic ataxia 4 (SPAX4)	MTPAP
Spastic ataxia with leukoencephalopathy (SPAX3)	MARS2
SPG11	SPG11
SPG15	ZFYVE26
SPG20	
SPG21	ACP33
SPG26	
SPG27	
SPG30	KIF1A
Spinocerebellar ataxia with axonal neuropathy	TDP1/Tyrosyl-DNA phosphodiesterase 1
Triple H syndrome	SLC25A15
Type III 3-methylglutaconic aciduria	OPA3
Vanishing white matter leukodystrophy	EIF2B1, EIF2B2, EIF2B3, EIF2B4, EIF2B5

Tabla 2. Dominant Ataxias

Disease	gene	Disease	gene
SCA1	ATXN1	SCA24	
SCA2	ATXN2	SCA25	
SCA3	ATXN3	SCA26	
SCA4		SCA27	FGF14
SCA5	SPTBN2	SCA28	AFG3L2
SCA6	CACNA1A	SCA33	
SCA7	ATXN7	SCA34	
SCA8	ATXN8	SCA35	TGM6
SCA9		SCA36	NOP56
SCA10	ATXN10	SCA37	
SCA11	TTBK2	SCA38	
SCA12	PPP2R2B	SCA39	
SCA13	KCNC3	SCA40	
SCA14	PRKCG	SPG7	
SCA15	ITPR1	Adult onset Alexander disease	GFAP
SCA16			
SCA17	TBP		
SCA18			
SCA19	KCND3		
SCA20	260-kb dup11q12		
SCA21			
SCA22	KCND3		
SCA23	PDYN		

HYPOTHESIS

The hereditary ataxias are a highly heterogenic disorder that in most of the cases affect mitochondrial function directly or indirectly. This mitochondrial dysfunction might affect cell viability with different trends depending on the genetic alteration responsible for the pathology and metabolic and immune status of the patient.

OBJECTIVES

1) To develop a diagnostic platform for the study of the genes responsible of ataxias by Next Generation Sequencing (NGS)

2) To determine the responsible mutation for the genetic ataxia using this platform in a group of ataxic patients without genetic diagnosis.

3) To validate the responsible mutation by testing genetic variables (location of mutation in the gene, frequency in the reference population, segregation in other affected cases, and absence in non affected relatives) and performing functional studies of the mutated protein.

4) To assess in cultured fibroblast the alterations of mitochondrial function produced by different genetically characterized heredoataxias looking for a unifying model of ataxias.

The achievement of these objectives will allow:

1) To reduce the diagnostic pilgrimage of patients with ataxia by accomplishing a precise diagnostic of these patients in a very short time frame, with the consequent cost savings.
2) In many cases it will be possible a personalized care of the patients based on the mutated gene finding.

These objectives are realistic since:

1) We have the necessary infrastructure (a NGS platform Ion Torrent)
2) The design of the gene panel showed a high coverage (more than 90%) of the regions of the ataxia genes.
3) There are similar projects to this proposal that are currently being carried in other centers abroad (see ref. Nemeth et al., Brain Oct. 2013).

Métodos, incluyendo análisis de datos (máximo 2 páginas)

Patients, controls, sample size:
This is a prospective study that includes 48 patients with ataxia from the Neurology Outpatient Clinic of the Hospital Ramón y Cajal that have excluded non-genetic causes (infections, demyelinating diseases, vascular, toxic, vitamin deficiencies, tumors and paraneoplastic) and therefore have a presumably genetic cause. We include in this study 10 patients with already known genetic cause, to check up the quality of the mutation detection test. For mitochondrial function studies we include a control group of 30 healthy volunteers. We have chosen this sample size because HaloPlex plates have 48 wells, which allow to study the sample in 1 plate optimizing the cost of the project. With this number, you will get 2-4 groups of 5-10 cases each with different types of mutations in each group (polyglutamine, alterations in mitochondrial DNA formation, channelopathies,..) to be compared with the control group. In all cases the participants will get a Project informed consent approved by the Ethics Committee of Hospital Ramón y Cajal.

Part 1: Determination of the mutation that causes ataxia

1) Sequencing of candidate genes for ataxia in nuclear genome.
The workflow sequencing of genes includes two phases, generation of the library and proper sequencing.
To generate the library we use 200 ng of DNA from each patient with the kit HaloPlex Target Enrichment System (Agilent Technologies) of 48 samples and capable of capturing 250 Kb to 2.5 Mb sequence. With the Haloplex kit the total region to capture in this experiment for subsequent sequencing is 715 Kb in size with a coverage of 99.85%, corresponding to the coding regions of candidate genes for dominant and recessive ataxias (Tables 1 and 2). The massive sequencing of genes, is performed on a system Personal Genome Machine (PGM) Ion Torrent (Life Technologies), a device based on a technology fast, simple, very efficient in terms of cost and productivity. To determine the type of chip that you need to use (314, 314, 318), it is calculate the theoretical depth of sequencing required in this experiment. We chose the chip 318 that has a capacity of 800 Mb sequence and sequencable region of this experiment is 715 Kb in size by the patient, obtaining a theoretical depth of 1118X (800/0.715). In this experiment, 5 samples can be loaded per chip, resulting in a theoretical depth of 223X (1118/5). An entire experiment is running 10 templates and 2, 318 sequencing chips. In sequencing using the Ion PGM 400™ Sequencing Kit for sequencing fragments of 400 bp average size and 318 Chip Ion v2 Kit.

2) Analysis of mitochondrial DNA
Mitochondria are extracted from blood cells by differential centrifugation and later on DNA extraction with Quiagen Midi Extraction kit. The workflow massive sequencing of mitochondrial DNA from each patient includes two phases: the generation of libraries and sequencing.
To generate the library we use of 100 ng of DNA extracted from each patient. The protocol consists of four phases: 1 - mitochondrial DNA digestion each patient using the Ion Xpress ™ Plus Fragment Library Kit, 2 - ligation of the adapters ends repair and hybridization with the bar codes using the Ion Xpress Barcode Adaptors 1 - 16 kit (Life Technology), 3 - size selection of DNA fragments using E-Gel ® SizeSelect ™ 2% Agarose, and 4 - PCR amplification of DNA targets using Platinum PCR SuperMix High Fidelity kit.
The massive sequencing of genes is performed in a system Personal Genome Machine (PGM) Ion Torrent (Life Technologies). To generate the template we use the Ion OneTouch ™ Template Kit 200 v2DL, starting from 20 ul of pooled libraries each patient to a molarity of 18 pM. This phase consists of an emulsion PCR in Ion One Touch System and an enrichment of the sample in the Enrichment System. Once generated, the template quality control is done with the Ion Sphere ™ Quality Control Kit 2.0 Qubit fluorometer (Invitrogen).
To calculate the theoretical depth sequencing necessary in this experiment, we use the 316 chip since it has a capacity of 200 Mb sequence and the region of this experiment is ~ 16 kb (mitochondrial DNA) per patient, obtaining a theoretical depth 12500X . Therefore, in this experiment can be loaded 12 samples per chip, resulting in a theoretical depth of 1000X. In sequencing using the Ion PGM ™ 200 Sequencing Kit for sequencing fragments of 200 bp average size and the chip 316

Part 2: Study of mitochondrial function

1. Obtaining cultured fibroblasts
The fibroblast cultures will be obtained from skin biopsies from patients with different types of ataxia, and age-matched control subjects using the methodology previously described (Johnson et al., 1990). Briefly, skin explants incubated at 37 ° C in Eagle's minimal essential medium (MEM) supplemented

with 10% fetal bovine serum, nonessential amino acids and antibiotics. When cells have been grown (about two weeks), they are washed with sterile PBS and 2ml of trypsine. Once dispersed, they will be grown in Petri dishes of 100-mm using the same culture medium as described above until reaching confluence. The purity of fibroblast cultures is checked by vimentin expression determined by immunocytochemistry. Before starting the various treatments, cultures will be deprived of fetal bovine serum reducing its concentration at 0.1% for 24 or 72 hours.

2. Study of mitochondrial function and generation of free radicals (ROS)

Mitochondrial membrane potential will be determined in cultured fibroblasts by flow cytometry (FACS). For this purpose, we will use commercial kits Invitrogen that allow the identification of three specific mitochondrial markers: 1) functional mitochondria (Mitotracker deep red) 2) Total mitochondria (Mitotracker green), and 3) ROS generating mitochondria (MitoSOX). The determinations will be carried out in cultured fibroblasts at baseline, and in a situation of oxidative stress induced by inhibiting the activity of complex I, II and III of the respiratory chain rotenone (10 uM), threnoyltryfluoacetona (TTFA, 10 uM) and antimycin A (40 ug / ml), respectively (Zhou et al., 2011). The activity of complex IV (COX) of the respiratory chain is determined as described in Rustin et al. (1994).

The determination of total glutathione levels and GSH will be done following the method described by Tietze (1969). Briefly, fibroblast cultures are washed in PBS and lysed in 100 ul of perchloric acid (PCA, 0.4N) for 30 min at 4 ◦ C, and centrifuged. The supernatants are neutralized with 4 volumes of NaH2PO4 (0.1 M), EDTA (5 mM), pH 7.5. GSH content is measured on an automatic plate reader by adding DTNB (0.6 mM), NADPH (0.2 mM) and glutathione reductase (1 U). The reaction is monitored at 412 nm for 6 minutes. In addition, glutathione determinations will be made by HPLC using the methodology described previously (Rodriguez-Martin et al., 2000).

3. Quantifying mitophagy

Mitophagy rate at different culture conditions will be studied using confocal microscopy assessing the percentage of total mitochondria (Mitotracker green-positive) that colocalize with associated protein LC3 autophagy. In parallel, we will determine by Western blotting the expression of beclin 1 and ATG5.

4. Inflamasomas formation and caspase-1 activity

To assess whether in fibroblasts from patients with ataxia activation induces ROS NLRP3/caspasa-1 we will study NLRP3 cellular localization by immunocytochemistry and active caspase-1 by detecting its irreversible inhibitor YVAD (biotinyl -YVAD-CMK, AnaSpec) previously added to the cultures (Herranz et al., 2012). In parallel, we will determine the rate of mitochondria producing ROS (MitoSOX) relative to caspase-1 activity by FACS.

5. Rating cell death in cultured human fibroblasts

To determine DNA fragmentation characteristic of apoptosis in the cultures we will use TUNEL technique (Lopez-Toledano et al., 2004). Furthermore, we will consider the fibroblast cell cycle, and the molecules involved in cell death (Fas, FasL, Bcl-2, p53, etc.) using FACS (Sanchez Torres and Vargas, 2003). Caspase-3 and caspase-9 levels we will measure by Western blot on cultures. Necrosis rate in the cultures will be determined by assessing the lactate dehydrogenase activity (LDH).

Data analysis

Data analysis will be performed at our center from basic bioinformatics (alignment detection, variants) to the filtering and selection of variants obtained clinic. The sequencing data is aligned to the reference genome UCSC hg19 in nuclear genes and mitochondrial DNA is aligned to the reference genome revised Cambridge Reference Sequence (rCRS) (GenBank accession number NC_012920) using the alignment algorithm Burrows-Wheeler Aligner (BWA). Variants are called with the Genome Analysis Toolkit (GATK) and annotated with Annovar software. Immunocytochemical analysis will be conducted using a NIKON confocal microscope coupled to C1-plus NIS program. The results will be expressed as the mean ± SEM of four independent experiments per patient, performed in triplicate or quadruplicate. The statistical analysis for immunocytochemical and biochemical studies will be performed using the Student t test or by one-way ANOVA followed by Newman-Keuls test for multiple comparisons. Differences are considered significant at $p < 0.05$.

-Plan de trabajo y cronograma (máximo 1 página)

The project will take place in the Hospital Ramón y Cajal.

The visit of patients, sampling and informed consent will be done in the Neurology Outpatient Clinic

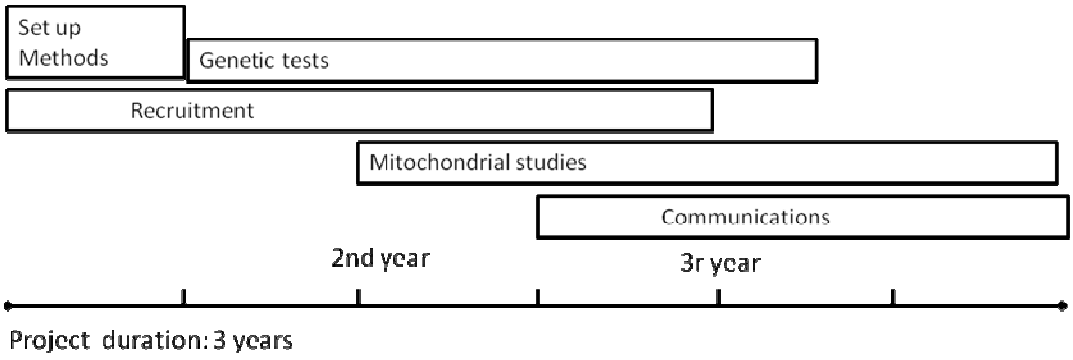
DNA extraction and sequencing of candidate genes and mitochondrial genome will be held at the Unidad Central de Apoyo para Estudios Genómicos del IRYCIS-Hospital Ramón y Cajal.

The study of mitochondrial function will be in Servicio de Neurobiología-Investigación del IRYCIS-Hospital Ramón y Cajal.

The stages of the study are shown in Figure Annexed. A graphic timeline is also included.

No additional staff is requested in the project. There are 2 laboratory technicians to carry out the study.

ACTIVITY / TASK	Delete line	STAFF INVOLVED		MONTH J	F	M	A	M	J	J	A	S	O	N	D
Selection of patients, informed consents		A. Jimenez Escrig	1st Year	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
			2nd Year	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
			3rd Year	✓	✓	✓									
ACTIVITY / TASK	Delete line	STAFF INVOLVED		MONTH J	F	M	A	M	J	J	A	S	O	N	D
Nuclear Gene and mitochondrial genome sequencing		G. Muñoz Martin A. Sanchez H. A. Jimenez Escrig	1st Year			✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
			2nd Year	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
			3rd Year	✓											
ACTIVITY / TASK	Delete line	STAFF INVOLVED		MONTH J	F	M	A	M	J	J	A	S	O	N	D
Fibroblast culturs		M.J. Casarejos D.Reimers E. Bazán	1st Year												
			2nd Year	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
			3rd Year	✓	✓										
ACTIVITY / TASK	Delete line	STAFF INVOLVED		MONTH J	F	M	A	M	J	J	A	S	O	N	D
Mitocondrial function evaluation, ROS and mitophagy studies		M.J. Casarejos D.Reimers E. Bazán	1st Year												
			2nd Year	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
			3rd Year	✓	✓	✓									
ACTIVITY / TASK	Delete line	STAFF INVOLVED		MONTH J	F	M	A	M	J	J	A	S	O	N	D
Inflamosomes and caspase-1 activity		M.J. Casarejos D.Reimers E. Bazán	1st Year												
			2nd Year						✓	✓	✓	✓	✓	✓	✓
			3rd Year	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓



Equipo investigador (máximo 2 páginas): presentación de los miembros del equipo especificando su cualificación, experiencia y capacidad para llevar a cabo los objetivos del proyecto.

RESEARCH TEAM:

Adriano Jiménez Escrig, MD PhD (Principal Investigator)
Antonio Sánchez Herranz, BS PhD
Eulalia Bazán Izquierdo, BS PhD
Diana Reimers Cerdá, BS PhD
M. José Casarejos Fernández, BS, PhD
Gloria Muñoz Martín, BS PhD

LOCATION: Servicio de Neurología, Hospital Ramón y Cajal and Servicio de Neurobiología-Investigación, y Unidad Central de Apoyo en Genómica Translacional del Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS). Madrid, España.

The research team has experience in reaching agreements with biotechnology companies for the exploiting the results, patent applications, etc. Regarding the dissemination of results the group maintains a continuous activity publications as well as participation in national and international conferences on degenerative diseases of the nervous system. The IP (Dr. Jiménez Escrig) participate regularly at training courses, conference presentations, lectures and dissertations. The IP is responsible for the consultation of Neurogenetics in the Neurology Service of the Hospital Ramón y Cajal, performs clinical and molecular diagnosis of ataxias, hereditary dementias, familial amyloid polyneuropathy and cerebrotendinous xanthomatosis in the center. He is expert in training on Neurogenetics, having tutored three PhD thesis on the subject (Alzheimer's Disease: Genetic Risk Factors for Alzheimer's Disease: Clinical and Epidemiological Study. Universidad de Alcalá, 2001, Dr. Baron; Study familial Parkinson's disease with high-density SNPs. Universidad de Alcalá 2009, M. Sagrario Manzano and cerebrotendinous xanthomatosis in Spain: mutations, clinical and therapeutic aspects. Universidad de Alcalá 2010). This late was awarded with the Prize Alberto Rabano 2010 for the best thesis in Neuroscience. He is also director of the PhD courses: Basic and Clinical Neurogenetics, years 2002-2009. University of Alcala and Short Course on Neurogenetics, a short course aimed to train European young Neurologists.

Dr. Sánchez Herranz is an expert in cell biology studies regarding mitochondrial activity and ROS toxicity in neurodegeneration. He is currently the Manager Director of Unidad Central de Apoyo en Genómica Traslacional del IRYCIS-Hospital Ramón y Cajal.

Dr. Gloria Muñoz has an extensive experience as a specialist in Molecular Genetics collaborating with several research groups in different genetics laboratories throughout his career. During his postdoctoral training worked on projects to unravel the genetic basis of complex traits such as obesity, cancer and aggressive behavior in the University of Chapel Hill, North Carolina (USA). Currently she is in charge of the Unidad Central de Apoyo en Genómica Traslacional del IRYCIS-Hospital Ramón y Cajal where we will run the Next Generation Sequencing experiments.

Dr. Diana Reimers, Dr. Eulalia Bazán and Dr. M. Jose Casarejos belong to the Neurobiology Department of the Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), whose line of research is in the field of Cell Therapy and Regenerative Medicine. Dr. Reimers and Dr. Bazán posses high experience in identifying factors which promote proliferation, migration and neuronal differentiation of the stem-cells, and in studying its therapeutic potential in experimental models of neurodegenerative diseases. Dr. Casarejos is expert in cell biology studies regarding mitochondrial activity and ROS toxicity in neurodegeneration

The group has published four papers in international journals in 2010 on neuropsichiatric disease genetic studies, 5 items in 2011 and now has 2 published articles on next generation sequencing in 2012, and additional two articles are under review and a chapter book. Currently, they have 4 complete genomes and 28 exome in patients with different neurological diseases, being the Spanish center with more clinical experience in this area (see ref. 10-11).

The IP currently manages a multidisciplinary reference Unit for Ataxia and Spastic Paraplegia in the Hospital Ramón y Cajal. The group has an extensive research experience in experimental models of ataxia. Recently we have published an article in Stem Cells International on the benefit of the transplantation of human umbilical cord blood stem cells in an experimental model of ataxia. There are two submitted manuscripts on the same field and an additional one in preparation (detailed in CV).

There is also a training line of ataxia, with a PhD work on preparation (Lucia Calatrava Ferreras, Universidad Autónoma de Madrid, Facultad de Biológicas) tutored by Dr. Bazán and Dr. Sanchez Herranz, and several practicum and end of master projects on this line.

Medios disponibles y medios solicitados a la Fundación para realizar el proyecto

We have the following media for carry on the project.

Clinical office with equipment, 2 PC computers with printer where we will perform the clinical assessment of affected, data collection and informed consent

Laboratory for molecular biology with two thermal cyclers two micro centrifuges, two power supplies and electrophoresis tank, bathroom, oven, microwave, etc. ..

DNA extraction and NGS with PGM (Ion Torrent) will be held at the UCA-GT at Ramón y Cajal Institute of Health Research (IRYCIS).

The UCA-GT has been created entirely new in July 2012 and is fully equipped for next generation sequencing techniques offered by the unit. Laboratory area 1 has been designed to make it the automated extraction of nucleic acids, nucleic acid analysis and automated preparation of libraries intended for the sequencing service. The laboratory area 2 is divided into two zones, one main area to service NGS, and other office area with workstations for bioinformatics analysis. The UCA-GT has an automated nucleic acid extractor Chemagic MSM I (Chemagen, PerkinElmer) spectrophotometer Nanodrop 2000 an Agilent 2200 Bioanalyzer System TapeStation a Qubit 2.0 Fluorometer a massive sequencer Personal Genome Machine (PGM) (Ion Torrent), an Ion Enrichment System and One Touch System. The UCA-GT features itself with the following small item: laminar flow cabinet with UV light for PCR, several microcentrifuge, centrifuge plate, vortex stirrer, pH meter, two thermal cyclers, 3 sets of micropipettes, heat block and equipment electrophoresis, refrigerators and freezers.

For the study of mitochondrial function with have:

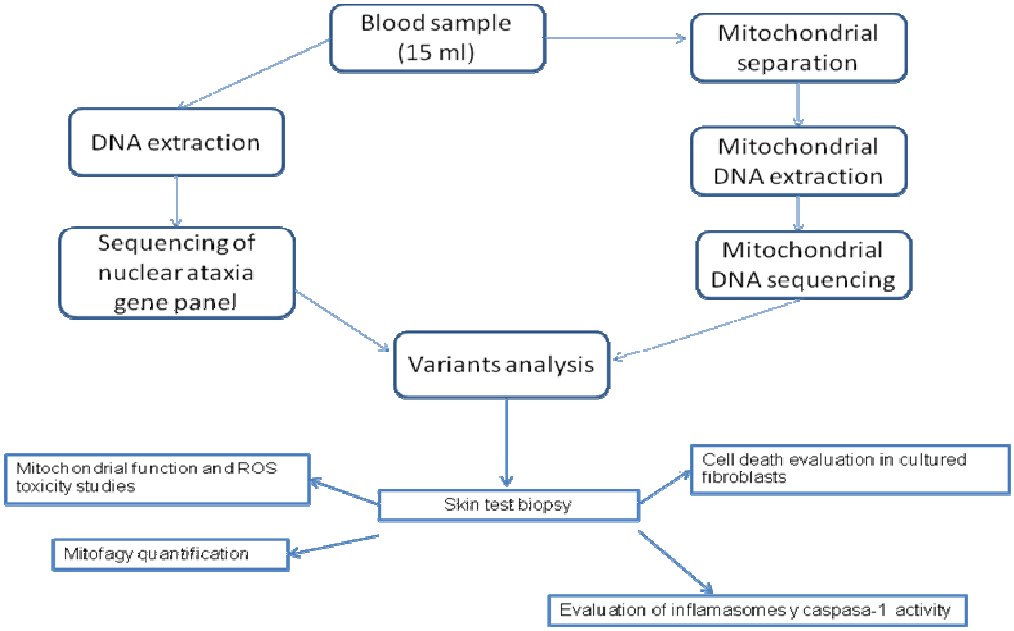
Two Cell Culture Units, Spectrophotometry Unit, Centrifugation and Ultracentrifugation and Proteomics Unit (HPLC, FPLC and Maldi-Toff and Western blot)

HPLC for determination of amino acids, catecholamines, glutathione, vitamins and other molecules

Flow Cytometry Unit, Conventional and Fluorescence Microscopy Unit

Confocal Microscopy Unit

Chart flow of the study



Limitations of the study

The study presents two possible limitations. The first is that a patient might have a disease-causing mutation in a gene is not known at present. For this type of patient we consider an exome study in an additional project if the family structure allows it. The common perception today is that the exome contains 85% of the disease-causing mutations. The second limitation is that in mutations produced by pathological repetitions may not be detected due to limitations on the technique used. This is a pilot project to assess these limitations, and in those cases where no gene can be examined by this technique we will use PCR and fragment analysis or other techniques to complete the information.

Cost justification

The project cost estimation includes an expense for the project expenditure necessary reagents for DNA extraction and sequencing of the genes involved in ataxia.

Experimental design-workflow for PGM sequencer (Ion Torrent)

- Expected coverage in the experiment and type of chip used: Intended sequence in 48 patients, the coding region of 85 genes related recessive ataxia (62 genes) and dominant ataxias (23 genes). 1,327 sequencing include regions corresponding approximately 289 Kb of sequence per sample. Haloplex kit with total sequence amplicons would be 25,359 and the region of 715 kb would be the sequence size per sample with a coverage of 99.85%. At the same time, it aims to sequence the mitochondrial DNA of these same 48 patients. Sequencing includes the 16 Kb in size of mitochondrial DNA per sample.

Considering the total cost of the project, each patient studied include sequencing of all candidate genes for ataxia and sequencing of their mitochondrial DNA (Tables 1 and 2) would cost about 779 euros, and in the following cases this price would be lower as it includes the costs involved to develop a technique. Once this technique is tuned it can be used to study any patient with genetic ataxias with a very affordable cost per test and overall giving the results in a few days.

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