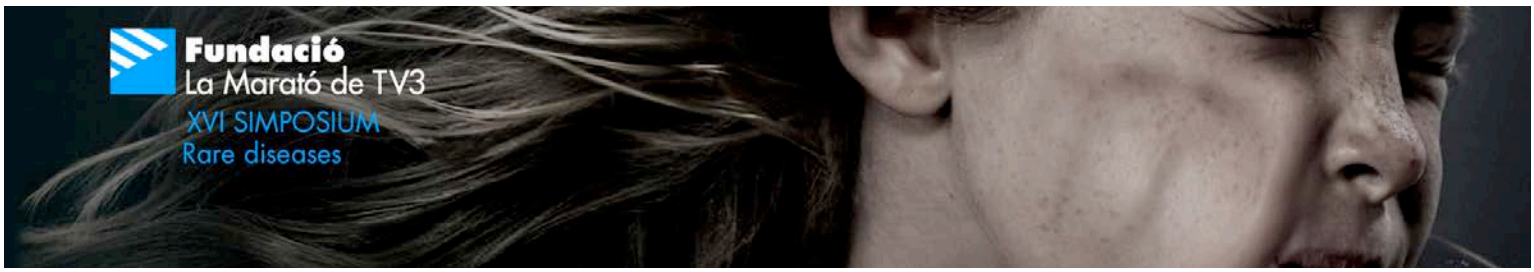


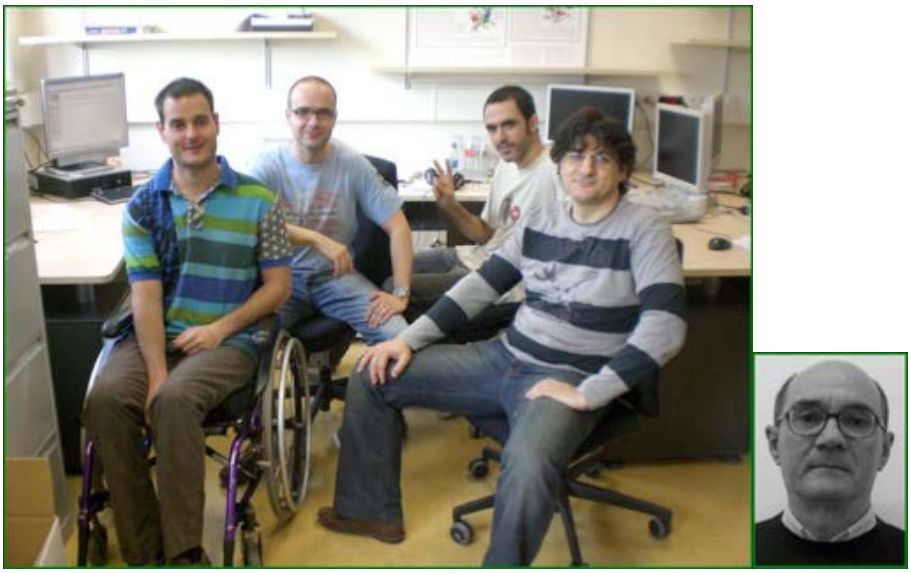
Friedreich's ataxia integrative research: Pathophysiological and therapeutic approach (FAIR)

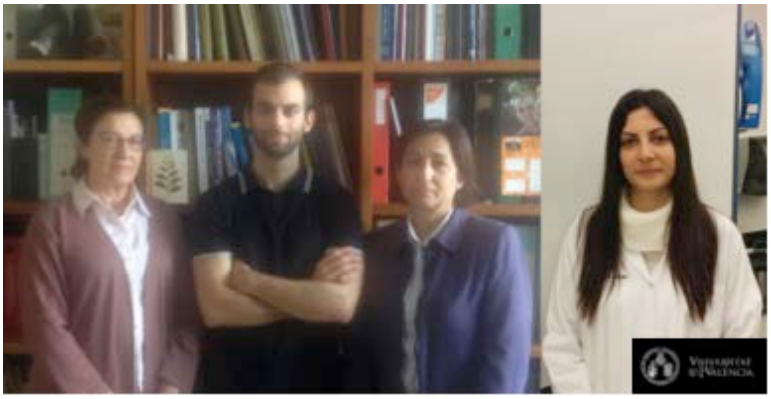
Dr Francesc Palau Martínez
CIBER Centro de Investigación Biomédica en Red,
Institut Biomedicina de València CSIC

Dr Enric Querol Murillo
Institut de Biotecnologia i Biomedicina.
Universitat Autònoma de Barcelona

Dr Maria Dolores Moltó Ruiz
Facultat de Ciències Biològiques. University of Valencia

Dr Jordi Tamarit Sumalla
Facultat de Medicina. Universitat de Lleida (UdL)






GAME
OVER

1. Summary

Since the identification of *FXN* as the mutant gene responsible for Friedreich's ataxia (FRDA, OMIM # 229300, ORPHA95) and for the protein which it encodes, frataxin, a lot of work has been done to understand the function of frataxin and the pathophysiology of the disease. The main objective of this project is to study the consequences of frataxin deficiency to try to discover the main affected cellular processes, using both biological models and bioinformatic tools. Knowledge generated by these complementary approaches has been the basis for search and validation of new biomarkers that may possibly be useful for diagnosis, clinical follow-up and clinical trials.

A Friedreich's Ataxia Integrative Research Consortium (FAIR) was created to reach this general aim. The FAIR Consortium, comprising four research groups, addressed this issue via four work packages: cellular physiopathology, function of frataxin, modifier genes, and biomarkers and drug screening.

2. Results

Knowledge of the molecular function of this protein has been obtained through the use of animal and cellular models. However, research in primary cells of tissues or organs primarily affected is strictly necessary. We proposed the use of cultured cell lines (cardiomyocytes, the neuron-like neuroblastoma cell line SH-SY5Y), primary cell cultures of dorsal root ganglia (DRG) and *Drosophila melanogaster* fly to understand the pathophysiological effects of frataxin deficiency. In addition, we made a global approximation of the molecular pathways affected in the biochemistry and biology of frataxin that allowed the identification of new drug targets for therapy of this disease.

Workpackage 1: Cellular Pathophysiology

The laboratories of the groups of the University of Lleida and the Institute of Biomedicine of Valencia (CSIC) worked with cellular models in which the expression of *FXN* gene had been silenced, either in primary culture of cardiac cells or human neuroblastoma SH-SY5Y cells. We also studied the effects of frataxin deficiency in the mouse model of FRDA, YG8R.

We developed new cell models from primary cultures of cardiomyocytes and neurons of the dorsal root ganglion from neonatal rats. Frataxin expression in these cells was interfered using shRNA transduced by lentiviral particles. The interference was verified by Western-blot and quantitative RT-PCR. The decreased expression of frataxin in cardiomyocytes involved a distinct change in the distribution pattern of the mitochondrial network, increased sensitivity to oxidative stress and increased presence of oxidized proteins. A distinct change in lipid metabolism, which leads to the accumulation of lipid droplets in frataxin-deficient cardiomyocytes was also observed. Moreover, metabolomic studies have identified that frataxin-deficient cardiomyocytes show clear metabolic disorders, and have also identified some potentially altered compounds. A proteomic analysis has also helped identify several altered proteins. These targets are currently being validated by targeted proteomics. With regard to the DRG neurons, a significant decrease was found in survival at 5 days from frataxin interference. This death is apoptotic. Moreover, the presence of neurite thickening that may be related to an alteration of the cytoskeleton was observed. Cell viability can be recovered by treatment with TAT-BH4 antiapoptotic peptide, opening the door to other potential treatment that improves the neurological symptoms of patients with Friedreich's ataxia. Another interesting aspect is the ratio of frataxin to calcium metabolism, since in this cell model a marked alteration was observed in the levels of this ion, as was also the possible beneficial effect on cell viability of calcium chelators.
We developed and functionally characterized a chronic disease model with RNA interference technology by generating stable frataxin-deficient lines in the human neuroblastoma line SH-SY5Y. We investigated the cellular and mitochondrial

consequences of the lack of frataxin in this neuron-like model and in sensory neurons of the dorsal ganglia of the YG8R mouse. In SH-SY5Y cells frataxin deficiency causes slower growth associated with cellular senescence. We have shown that reducing frataxin induces mitochondrial dysfunction due to a bioenergetic deficit and improper metabolism and Ca^{2+} in mitochondria that is associated with reticulum stress and oxidative stress. In addition, frataxin depletion does not cause cell death but increases autophagy, which may have a cytoprotective effect against damage such as oxidative stress. Primary culture studies have shown DRG cytoskeletal alteration with the formation of abnormal structures associated with neurodegeneration in the axons of neurons (beadings). This could be due to altered of Ca^{2+} metabolism, also confirmed in this neuronal model.

Pathways associated with neurodegeneration that may be involved in the pathophysiology of Friedreich's ataxia were studied. For this, the expression of proteins related to oxidative stress, apoptosis, autophagy, and energy metabolism was investigated in different mouse tissues YG8R: dorsal root ganglia, nerve roots, posterior columns and brainstem. We noted that the nerve roots and DRGs are more affected than other neural tissues. This suggests that the axonopathy may be the primary defect in the YG8R mouse, with DRG and column degeneration being the result of a neuropathic dying-back process.

Workpackage 2: Frataxin function

To understand the mechanisms leading to the accumulation of iron in the absence of frataxin and define the role of this protein in iron metabolism the laboratory of the University of Lleida worked with a frataxin-deficient yeast model. Strains were used in which expression of frataxin yeast (YFH1) was under control of the Tet promoter, repressible by the addition of doxycycline to the culture medium. Proteomic analysis of these mutants after the repression of YFH1 showed us Adh2 content reduction and ALD4. Since these two proteins are under the transcriptional regulatory control of ADR1, a transcriptome study was conducted,

showing that many other ADR1-dependent genes were repressed. ADR1 labelling with GFP protein led to the discovery that the cellular localization of ADR1 changes after the suppression of YFH1. Also, transcriptome analysis allowed the observation that several iron regulon genes were induced, which would explain the accumulation of iron in YFH1-deficient mutants. This led us to study the role of Cth2, a member of the iron regulon which causes metabolic remodeling by reducing the content of a number of iron-dependent proteins. Analysis of Cth2-deficient mutants verified that this protein is responsible for the decrease in content and activity of aconitase, succinate dehydrogenase and cytochrome *c* when YFH1 is absent. This process explains deficiency of proteins with iron-sulfur centers (ISC) observed in the absence of frataxin.

For a better understanding of the relationship between structure and function of frataxin other proteins that interact with frataxin were identified using in silico tools. The DockAnalyse algorithm was designed and applied to modeling the complex in which frataxin and its partner proteins are involved to propose the dynamics of ISC formation in that complex. We also studied the relationship of frataxin with moonlighting proteins due to the multiple functions that have been proposed so far. In addition, there has been an improvement in several respects of the bioinformatics tool for analysing gene expression networks developed in the laboratory of the Autonomous University of Barcelona. We have also identified different candidate genes to be studied in depth and proposed different substances to be tested in model organisms available to other groups of the project.

Workpackage 3: Gene modifiers

Using a model of Friedreich's ataxia in *Drosophila*, which was obtained in the laboratory of the University of Valencia, modifier genes have been sought for phenotypes associated with loss of frataxin function. The model flies have significantly reduced survival and motor skills, exhibit accumulation of mitochondrial Fe, and are very sensitive to oxidative stress. These phenotypes

have been described in other models of FRDA and show great similarity to the clinical picture in patients. By screening candidate genes we identified modifiers that partially or fully recover the climbing ability of frataxin-deficient flies. These modifier genes are grouped into two functional types: (i) genes involved in metabolism of metals; (Ii) biochemical pathway components of TOR (target of rapamycin) signaling pathway conserved in evolution and involved in processes that allow the control of cell homeostasis in response to their environment. Specifically, reducing the expression of genes coding for transporters of Fe, Cu and Zn improves flies' motor skills. The reduced activity of TOR complex 1 (TORC1) has a beneficial effect on the model, significantly improving flies' climbing ability and survival. This result was key for including the compound rapamycin in all drugs tested, since rapamycin is a chemical inhibitor of TORC1. As expected, rapamycin treatment improves the phenotypes of the model flies. We have identified that this beneficial effect is provided by greater protection against oxidative stress generated by frataxin deficiency. This additional protection is obtained by means of the Cnc transcription factor that translocates into the nucleus after treatment with rapamycin, thus increasing the expression of a battery of antioxidant genes regulated by Cnc. As a result, several biochemical markers of oxidative stress regain normal level. Furthermore, when the model flies are subjected to external oxidizing agents, rapamycin induces autophagy as a mechanism for controlling the levels of oxidative stress and thus recovery of enzyme activities that are key to the cell, such as aconitase activity, which is decreased in patients with FRDA.

Workpackage 4: Biomarkers and drug screening

Proteome analysis has been carried out in three models of frataxin deficiency in the laboratories of the University of Valencia and the Institute of Biomedicine of Valencia: *Drosophila* model, neuroblastoma cells and YG8R mouse dorsal root ganglia. The study in *Drosophila* allowed the identification of a total of 69 proteins that are expressed differentially in deficient flies compared to control flies in both normal culture conditions and strong oxidative stress. These proteins are involved in oxidative phosphorylation, cycle of tricarboxylic acids,

glycolysis/gluconeogenesis, metabolism of fatty acids, proteasome and iron homeostasis. This indicates that frataxin deficiency is accompanied by alterations in a number of metabolic pathways. In the chronic model of neuron-like neuroblastoma cells 35 proteins related to protein processing in the endoplasmic reticulum, autophagy regulation, regulation of the actin cytoskeleton, calcium signaling pathways, oxidative phosphorylation and cell cycle were identified, supporting many of the previously described results. Finally, in the study of the protein profile of YG8R mouse dorsal root ganglia compared to C57BL/6J control strain, 329 proteins were identified. Some of these routes are: proteolysis mediated by ubiquitination, proteasome generation, protein processing in the endoplasmic reticulum, regulation of autophagy, regulation of the actin cytoskeleton, calcium signaling pathways, oxidative phosphorylation, axon guidance and insulin signaling pathway. This indicates that frataxin deficiency is accompanied by alterations in a number of metabolic pathways and that many of these routes are shared by the different models.

Using the model of Friedreich's ataxia in *Drosophila*, we tested the effect of 25 drugs on such phenotypes and we analyzed the effect of oxidative stress in the whole protein or proteome. The 25 tested compounds represent molecules already tested in humans, easily available on the market and whose mechanism of action could be reasonably useful for the treatment of FRDA. Among them, we found that a Cu chelator and another compound with TORC1 inhibitor activity allow the frataxin-deficient flies to achieve the climbing capabilities of control individuals. For the analysis of drug compounds in cultured DRG neurons we opted for studying drugs that would modulate calcium levels, as we had observed an incorrect buffering of calcium in this model. After noting that frataxin deficiency involves a defect in the activity of calpain that is directly related to the amount of frataxin present in the cell, a study of calpain activity was performed. After treatment with calcium chelators (EGTA and BAPTA) or inhibitors (o-phenanthroline) we observed a decrease in activity of calpain genotypes in

C57BL/6J (increased frataxin) while this difference is very mild in the YG8R mutant mice.

3. Relevance and possible implications

The bioinformatics research and the results obtained in our biological models have helped not only to understand the role and molecular properties of frataxin and the proteins with which it interacts, but also to increase overall knowledge of the molecular pathology of this disease.

One major aim of the work was to define new tools that facilitate the diagnosis and/or evolutionary follow-up of treatments in Friedreich's ataxia. To this end, we propose the search for biomarkers associated with frataxin deficiency through a proteomic and metabolomic analysis. The identification of a proteomic profile in different deficient disease models for Friedreich's ataxia has allowed confirmation of biological processes that had previously been associated with frataxin deficiency and others that had not previously been related. These findings increase the number of processes and metabolic pathways in which frataxin may be involved, and should allow further study in our models to better understand the pathophysiology of the disease. The final confirmation of these candidates as biomarkers requires tracking large numbers of patient samples in different stages of the disease.

The development of cellular models based on the tissues most affected in the pathology studied (heart and neurons of the dorsal root ganglia) and the *Drosophila* model makes it possible to test the possibilities of reversing the consequences of the lack of frataxin that can have multiple potentially therapeutic compounds. Our work has identified a possible beneficial of Cu chelators and TORC1 inhibitors, in particular rapamycin-related compounds. In addition, we identified the mTOR pathway as a new biochemical pathway associated with

frataxin deficiency, constituting a new therapeutic target. Likewise, the mechanisms described in this project deserve to be taken into account for exploration as potential therapeutic for the treatment of patients FRDA, such as manipulation of Ca^{2+} homeostasis and lipid metabolism, which should be explored as potential treatment target strategies. Finally, the bioinformatics tools developed for analysis of gene expression networks and the use of other existing bioinformatics tools have identified a number of potentially useful compounds as candidates for Friedreich's ataxia drugs.

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