# PRECLINICAL TESTING OF DRUGS AGAINST THE TOXIC EFFECTS OF MUTANT HUNTINGTIN IN MURINE MODELS OF HD

**Rafael Vázquez Manrique**
Hospital Universitari i Politècnic La Fe València

## 1. Summary of the project

Many neurodegenerative diseases such as Alzheimer, Parkinson and Huntington's disease (HD), show phenotypic traits that make them easy to differentiate clinically. However, they show a remarkable common characteristic: the presence of protein aggregates in the neurons. It is still unclear whether these aggregates are a cause or consequence of the disease. Regardless of its nature (that is, cause or consequence) the accumulation of such aggregates sequesters resources that would otherwise be used to buffer the malfunction of proteins. All living organisms naturally show an accumulation of protein aggregates which is age-dependent. However, in these diseases protein aggregation rate dramatically increases due mainly to the presence of agglutinating mutant molecules, which are often hereditary. The dynamics of the aggregation processes have an impact on several key cellular functions (intracellular transport, synaptic function, etc.) for the modulation of the rate of accumulation of toxic species. Although these mutant molecules are largely responsible for the aggregation phenotype, some modifiers can modulate the progression of aggregation. Since the activity of many of these modifying genes is susceptible to regulation by chemical compounds, this makes it possible to ectopically influence the rate of aggregation and progression of the disease. Our hypothesis is that there are many unknown protective genes, and that there are also chemical compounds that can be activated to protect the cell from toxic molecules. We have produced data that shows that AMPK activators are able to alleviate the phenotypes present in models of two neurodegenerative diseases: Huntington disease and Lafora (progressive myoclonic epilepsy produced by accumulation of insoluble polyglucosans and proteins in the brain and other tissues, which is fatal). For example, AMPK activators are able to reduce cell deterioration in murine and *C. elegans* models of HD. In addition, AMPK activators are able to relieve the phenotype of cells from patients and murine models of Lafora disease. We believe that the use of strategies to alleviate the malfunction of the proteins and the stress of the endoplasmic reticulum (ER) could be beneficial to HD and Lafora disease. Our data suggest that there are common mechanisms that allow cells to survive in both pathologies, and that this can be applied to other neurodegenerative diseases. The general objective of this proposal is to consolidate our knowledge about the benefits of the activation of the AMPK, as well as to investigate the mechanisms underlying these effects. AMPK is a master regulator of cellular energy homeostasis and also a therapeutic target to treat HD. In addition to maintaining energy levels

AMPK is able to fight diverse cellular stresses such as that induced by mutant huntingtin (mHtt). Thus, our hypothesis is that activating AMPK in mammals will reduce cellular toxicity caused by mutant protein and therefore we will be attacking the root cause of the disease. We believe that reduction of mHtt-induced toxicity may be enhanced through activation of autophagy and other mechanisms, such as reduction of inflammation. Our analysis used zQ175 mice, which is a well-defined model of HD. These mice express around 190 CAG triplets in the first exon of *HTT* and superbly summarize the neurodegeneration that is observed in humans. The main objective can be subdivided into three sub-objectives:

1-Testing possible drugs that can alleviate phenotypes related to HD in models of the disease in *C. elegans*. We used a *C. elegans* strain that expresses 40 polyglutamines (polyQs, 40Q) in muscle cells, which show a polyQ-dependent aggregation phenotype of age. We screened for AMPK activators and molecules that alleviate ER stress, which reduce or delay the formation of aggregates. As AMPK activators we tried metformin, trehalose, resveratrol, MVR1316, propranolol and epigallocatechin 3-O-gallate.

2-Preclinical metformin assay (AMPK activator) and stress reducers in ER in a HD model. We tried these compounds, either alone or in combination, to stop or slow the progress of the disease in these mice. As a proof of concept we began treatment with metformin, an AMPK activator widely used in the treatment of type 2 diabetes. Next, we used the most effective compounds tested in part 1. These compounds were supplied to 3-month-old mice and the treatment was extended for three months. The progression of the disease was measured after three months of treatment using standard behaviour tests. Finally, we assessed the presence of aggregates and signs of degeneration and cell death in post-mortem samples of rodent brain.

## 2. Results

### Objective 1) Work with invertebrate models

To make a first attempt with the drugs we used the *C. elegans* model of polyglutamine (polyQs) toxicity from the laboratory of Prof. Morimoto (i.e. worms that express 40Q::YFP in muscle cells). However, we also created our own model of polyQs toxicity in neural cells of *C. elegans* to test functional recovery of worms by the drugs. The

debate about whether the aggregates are cause or consequence of the disease is hot, so regardless of whether studying aggregation dynamics is interesting, we also wanted to test functionality of the nervous system. Our model consists of transgenic worms carrying a construct that expresses 112 CAG triplets in mechanosensory neurons. In these worms we tested various AMPK activators with very positive results. We investigated the following substances: metformin, metformin, phenylbutyrate, trehalose, MVR1316 (an AMPK synthetic activating compound synthesised by Dr Ana Castro of the Institute of Medical Chemistry-CSIC, Madrid), salicylate and bedaquiline, a drug used in the treatment of tuberculosis.

In addition to functional analysis in *C. elegans*, to rescue the worms from polyQ toxicity we conducted studies to verify potential mechanisms of action of these substances. For example, we have verified in a previous research article that the neuroprotective effect of metformin on worms stressed by polyQs is largely due to the AMPK enzyme. We believe this is because mutants that do not have the activity of this molecule are not capable of being rescued by this substance (Vázquez-Manrique *et al*., 2016, Hum Mol Genet). Following this logic we have verified that salicylate also needs AMPK activity to perform its protective function.

As we said, in addition to our polyQ model (112Q::TdTomato), we have used the *C. elegans* model of nematodes that expresses 40Q::YFP in body wall muscle to investigate whether metformin is able to modify aggregation patterns, and we have verified that metformin is able to reduce aggregates in these worms. In addition, we showed that aggregation is dependent on AMPK and autophagy. To prove that metformin needs AMPK activity we introduced a mutant allele of AMPKα (*aak-2*) into worms that expresses 40Q (40Q; *aak-2*). When we tested metformin in these worms, they did not show reduction of inclusion bodies. Also, when the test included chloroquine, a well-known inhibitor of autophagy, we observed that metformin was not able to rescue aggregation of polyQs, confirming that metformin activates autophagy. On the other hand, once we knew that MVR1316 is activating AMPK, we continued to investigate the potential mechanism behind neuroprotection exercised by this compound. One of the possible pathways is activation of autophagy, as happens with metformin. To detect activation of autophagy we used a strain of worms that express LC3 (essential for the first steps of autophagy. LC3 is called LGG-1 in worms) fused in frame with two fluorescent proteins. When autophagy is activated, LGG-1/LC3 protease

is digested to allow the formation of autophagosomes. The products of this cut can be followed by Western Blot, and gives a direct measurement of the degree of activation of autophagy. Using these animals, we observed that MVR1315 is able to activate autophagy, since it induces the appearance of digested products. In addition, we also noted that metformin is capable of inducing LGG-1 processing, so this substance activates autophagy, through AMPK.

**Objective 2) Work with the mouse model of HD, zQ175**

The main objective of the project was always to perform preclinical trials in HD mice models. Firstly we set up the proof of concept of the model zQ175. To do this we performed a series of behavioural experiments, which provided us with functional data for control and HD mice. In addition, we developed investigation of a series of molecular markers, tested after sacrificing our animals: markers of inflammation (GFAP and IBA1), mutant huntingtin (mHtt), a target of AMPK (pACC), the neurotrophic BDNF factor and an autophagy marker (p62). All of these biomarkers showed that activating AMPK reduces phenotypes of HD (reduction of inflammation, reduction of mHtt aggregates, increase of BDNF, etc.).

Regarding the behaviour test, we used the Rotarod, in which mice are subjected to a challenge on a cylinder that rotates, and in which the animals must maintain equilibrium. This experiment provides us with data on the animals' motor capacity. Another test is Beam Balance in which animals have to pass through a series of walkways of different width. Finally the animals are subjected to a stress test, which measures their mood. This tail suspension test consists in holding the animals upside down by their tails, and measuring the time that the animals keep on fighting to rise. When a mouse is depressed it stays longer without attempting to rise.
Using these techniques, we have tested various activators of AMPK in zQ175 mice: metformin, trehalose, resveratrol, MVR1316, propranolol and epigallocatechin-3-O-gallate. The first compound that we proposed in the initial memory, metformin, has proved to be a substance with a surprising neuroprotective capacity (Sanchis *et al*., Experimental and Molecular Medicine, in press).

MVR1316 is one of the strongest AMPK activators in cell cultures (100 times more than metformin), and hence it has an enormous power for neuroprotection in worms stressed by polyQs. Unfortunately, MVR1316 has a moderate capacity to rescue motor

coordination of zQ175 mice. Why is this? We believe this is an effect of the low solubility in water of MVR1316. This could make bioavailability of this compound low in the murine brain. In any case it is still protective so some of the product has reached the nervous system. In regard to this problem, our colleague, Dr Ana Castro from the IQM (CSIC, Madrid) has generated a new basic version of MVR1316, which seems more soluble. Although the experiments are in a preliminary state, we will continue developing these chemical derivatives, thanks to the grant given by the Fundación Ramón Areces (CIVP19S8119).

**Observational study in HD patients**

In addition to these objectives, we had the opportunity of carrying out an observational study with data from a database of HD patients, the Enroll-HD (https://www.enroll-hd.org/). Enroll-HD is a global observational clinical study that collects data from HD patients and healthy controls and includes very standardized data on aspects as diverse as genetics, patients' motor and cognitive capacity, medication, etc. (related to HD and other diseases).

Since type 2 diabetes is widespread worldwide (and metformin is taken to treat this condition), we hypothesised that a proportion of HD patients could be medicated with this drug. Hence, we studied how they behaved in relation to HD patients who did not take metformin. We conducted a statistical study of the cognitive ability of HD patients who took the substance compared with those who did not. Since type 2 diabetes is a disease that has a very negative impact on the nervous system, we used the cognitive ability of healthy controls with and without type 2 diabetes, to subtract this impact of HD patients. To test the cognitive ability of patients and controls, we used the scores of cognitive ability tests described in Enroll-HD: verbal fluency, Stroop interference, Symbol Digit Modalities, Trail Making tests, Stroop word reading and Stroop colour naming test. We also used a variable that encompasses all of these tests, the Cognitive Score. Statistical analysis included as covariates: body weight index, educational status, sex and age of the participants. The result of this regression analysis showed that in fact, people with HD who also take metformin to treat their type 2 diabetics generally showed better scores in cognitive tests. These results are published in Hervás *et al.*, PLOS ONE (2017). We believe that these data constitute a fundamental support for our preclinical experiments using metformin, and that they support future clinical

trials using this substance. In 2017 we obtained funding to carry out this clinical trial (PI17/00011).

## 3. Relevance and future implications

We have shown that AMPK is a powerful target to treat Huntington disease, and importantly its activity can be modulated with drugs such as metformin.
Thanks to the results of this project, it has been possible to consolidate a new proposal from the Instituto de Salud Carlos III (Ref.: PI17/00011; Ministerio de Ciencia, Innovacion y Universidades), which will allow to continue the work of the La Marató de TV· project. In this new project, we will conduct a double-blind, placebo-controlled clinical trial using metformin as a therapeutic agent against Huntington's disease. Metformin is not expensive and has few side effects. If this study works, we will expand the cohort and maybe in future doctors could prescribe metformin to patients of Huntington disease to delay the progression of the disease.

In this project from the ISCIII, we will also investigate the dual activation of AMPK with metformin and salicylate in a mouse model of HD. We have also achieved a project by the Fundación Ramón Areces (Ref.: CIVP19S8119), where we will investigate the activity of an activator of AMPK (MVR1316) that was synthesized by one of our collaborators, Dr Ana Castro (IQM-CSIC, Madrid), also in the model mice of the HD (zQ175). This molecule is property of Dr Castro, so we can patent the use of this product to treat HD.

With all this, we believe that the continuity of the work developed in the La Marató de TV· project is guaranteed, and we hope to bring neurologists new therapeutic strategies to be tested through clinical trials.

Moreover, we have begun to write a project to designate metformin as an orphan medication to treat HD. This would open the possibility of attracting the attention of the pharmaceutical industry to develop therapies against this devastating neurodegenerative disease. In this regard, thanks to the results of our project, we have been able to attract the attention of the Kaertor Foundation (kaertorfoundation.org), which promotes the interaction between basic groups and the

pharmaceutical industry, to establish alliances that bring the results of the bench to clinical trials. We are consolidating this collaboration, to bring the indoles (MVR1316) synthesized by our collaborator, Dr Ana Castro (IQM-CSIC, Madrid) to clinical trials.

## 4. Published data

**Publications in indexed journals**

Hervás D, Fornés-Ferrer V, Gómez-Escribano AP, Sequedo MD, Peiró C, Millán JM, Vázquez-Manrique RP*. (2017) Metformin intake associates with better cognitive function in patients with Huntington's disease. PLoS ONE 12(6): e0179283. (*corresponding author).

Sanchis A, García-Gimeno MA, Cañada-Martínez AJ, Sequedo MD, Millán JM, Sanz P*, Vázquez-Manrique RP*. Metformin treatment reduces motor and neuropsychiatric phenotypes in the zQ175 mouse model of Huntington disease. (Experimental and Molecular Medicine, in press) (*corresponding authors).

**Manuscripts in progress**

Bono-Yagüe J#, Gómez-Escribano AP#, Sequedo MD, Hervás D, Fornés-Ferrer V, Burguera J, Peiró C, Millán JM, Vázquez-Manrique RP*. Synergistic combinations of metformin and salicylate reduce polyglutamine toxicity in *C. elegans*. (*corresponding author; #equal contribution).

Sanchis A, García-Gimeno MA, Sequedo MD, Torres, S, Millán JM, Sanz P*, Vázquez-Manrique RP*. Activation of AMPK through substances of different nature reduces HD phenotypes in mice. (*corresponding authors).

**Congress communications**

Annual meeting of the CIBERER Network (2017). Nuevas aproximaciones terapéuticas a la Enfermedad de Huntington mediante el uso de modelos animales. Sanchis,A. García-Gimeno,M.A. Vázquez-Manrique,R. Sanz,P.

EHDN Plenary Meeting Vienna, Austria, (2018). Synergistic combinations of metformin and salicylate reduce polyglutamine toxicity in *C. elegans*. Bono-Yagüe J*, Gómez-

Escribano AP*, Sequedo MD, Hervás D, Fornés-Ferrer V, Burguera J, Peiró C, Millán JM, Vázquez-Manrique RP. https://jnnp.bmj.com/content/89/Suppl_1/A95.3.

CONBIOPREVAL meeting, Valencia, (2019). Metformin and salicylate alleviate polyglutamine aggregation by activating synergistically AMPK in *C. elegans*. Bono-Yagüe J*, Gómez-Escribano AP*, Sequedo MD, Hervás D, Fornés-Ferrer V, Millán JM and Vázquez-Manrique RP.

**Master thesis**

Title: Efectos de la combinación de metformina y salicilato en la toxicidad inducida por poliglutaminas en Caenorhabditis elegans

Student: José Bono Yagüe

Supervisor: Rafael Vázquez Manrique

Mark: Excellence with honors