UNIVERSIDADE DE COIMBRA

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TREHALOSE ALLEVIATES NEUROPATHOLOGICAL AND MOTOR BEHAVIOUR DEFICITS OF A TRANSGENIC MOUSE MODEL OF MACHADO-JOSEPH DISEASE

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Abbreviations

AD – Alzheimer's disease

Aβ – Amyloid beta

ALS – Amyotrophic lateral sclerosis

cDNA – Complementary deoxyribonucleic acid

CMV – Cytomegalovirus

DAPI – 4, 6-diamidino-2-phenylindoline

DNA – Deoxyribonucleic acid

DRPLA – Dentatorubral-pallidoluysian atrophy

DUB – Deubiquitinating

ERAD – Endoplasmic reticulum-associated degradation

HA – Hemagglutinin

i.p. – Intra-peritoneal

LC3 – Light chain 3

MAP2 – Microtubule-associated protein 2

MJD – Machado Joseph disease

MJDTg – Machado Joseph disease transgenic mice

MRI – Magnetic resonance imaging

MTOC – Microtubule-organizing center

mTOR – Mammalian target of rapamycin

NCI – Neuronal cytoplasmic inclusion

NES – Nuclear export signal

NGS – Normal goat serum

NII – Neuronal intranuclear inclusion

NLS – Nuclear localization signal

OPMD – Oculopharyngeal muscular dystrophy

PABPN1 – Polyadenylate-binding protein nuclear 1

PBS – Phospate buffer solution

PFA – Paraformaldehyde

PGK – Phosphoglycerate kinase

PolyQ – Polyglutamine

PrP^{Sc} – Proteinaceous infectious particle

RT – Room temperature

SBMA – Spinal bulbar muscular atrophy

SCA3 – Spinocerebellar ataxia type 3

SCA14 – Spinocerebellar ataxia type 14

SCA17 – Spinocerebellar ataxia type 17

SOD1 – Superoxide dismutase 1

SQSTM1/p62 – sequestosome 1/p62

Tg – Transgenic

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labelling

UIM – Ubiquitin-interaction motif

UPS – Ubiquitin-proteasome system

VCP – Valosin-containing protein

YAC – Yeast artificial chromosome

5-HT – 5-hydroxytryptamine

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Abstract

Machado Joseph disease, or spinocerebellar ataxia type 3 is a fatal neurodegenerative disorder considered the most common dominantly inherited ataxia worldwide. This disease is caused by an expansion of the CAG trinucleotide in the coding region of the *ATXN3/MJD1* gene, which is translated into an expanded polyglutamine tract in the C-terminus of the protein ataxin-3. This mutation of ataxin-3 enhances its susceptibility to misfold and accumulate as neuronal cytoplasmic and intranuclear inclusions, with a toxic gain of function, as it leads to neuronal dysfunction and cell death. Presently, there are no therapies able to modify or delay the progression of this fatal disease.

Trehalose is a naturally occurring disaccharide used in food, cosmetic and pharmaceutical industry as a stabilizer. Recent evidence indicates that trehalose acts as a chemical chaperone, stabilizing proteins and protecting them from denaturation and aggregation, and allowing the maintainance of its normal functions under environmental stresses. Moreover, trehalose has been shown to induce autophagy, a major clearance pathway for the degradation of dysfunctional proteins and organelles, and also important for the removal of intracellular aggregate-prone proteins as ataxin-3. Recent studies using *in vitro* and *in vivo* models of several protein-misfolded diseases, including spinocerebellar ataxias, present trehalose as a promising therapeutic approach.

In the present study we used a MJD transgenic mouse model to investigate whether trehalose can alleviate both motor behaviour and neurodegeneration characteristic of this disorder. We found that trehalose had a neuroprotective effect, improving significantly the extremely severe phenotype of these transgenic MJD mice. Trehalose ameliorated motor coordination and balance, and rescued limb and gait ataxia of treated mice. Furthermore, trehalose alleviated the neuropathology of this MJD mouse model, preserving both molecular and granular layers of the cerebellum, and reduced the size of mutant ataxin-3 aggregates in the Purkinje cells. Therefore, the low toxicity profile already described for this molecule, together with our results, suggest that trehalose can be a strong therapeutic approach for MJD.

Keywords:

Spinocerebellar ataxia type 3; Machado Joseph disease; Trehalose; Chemical chaperone; MJD transgenic mice

Resumo

A doença de Machado Joseph (MJD), também conhecida como ataxia espinocerebelosa do tipo 3 (SCA3), é uma doença neurodegenerativa fatal, considerada a ataxia espinocerebelosa dominante autossómica mais comum em todo o mundo. Esta doença é causada por uma repetição excessiva do trinucleótido CAG no gene ATXN3/MJD1, que se traduz numa cadeia expandida de glutaminas no C-terminus da proteína ataxina-3. Esta mutação da ataxina-3 aumenta a sua susceptibilidade para adoptar uma conformação alterada e para se acumular em inclusões neuronais citoplasmáticas e nucleares, adquirindo propriedades tóxicas, e originando disfunção neuronal e morte celular. Presentemente, não estão disponíveis terapias capazes de modificar ou atrasar a progressão desta doença fatal.

A trealose é um dissacarídeo de ocorrência natural usado nas indústrias alimentar, cosmética e farmacêutica como estabilizante. Estudos recentes indicam que a trealose actua como chaperone químico, estabilizando as proteínas e protegendo-as da desnaturação, da agregação, e permitindo a manutenção das suas funções normais quando sujeitas a stresses ambientais. Além disso, foi também demonstrado que a trealose induz a autofagia, uma via importante de degradação de proteínas e organelos disfuncionais, e que é também essencial para a remoção de proteínas intracelulares com tendência a agregar, tal como a ataxina-3. Estudos recentes *in vitro* e *in vivo* em modelos de diversas doenças neurodegenerativas, incluindo de ataxias espinocerebelosas, apresentam o tratamento com trealose como promissor.

No presente estudo usámos um modelo de murganhos portadores da MJD para investigar se a trealose poderia aliviar o comportamento motor e a neurodegeneração característicos desta doença. Foi demonstrado que a trealose teve um efeito neuroprotector, melhorando significativamente o fenótipo extremamente severo destes murganhos portadores da MJD. A trealose melhorou a coordenação motora, o equilíbrio, e a marcha atáxica dos animais tratados. Além disso, a trealose aliviou a neuropatologia neste modelo da MJD, preservando as camadas molecular e granular do cerebelo, e reduzindo o tamanho dos agregados contendo ataxina-3 mutante nas células de Purkinje. Em conclusão, o baixo perfil de toxicidade já descrito para esta molécula, em conjunto com os resultados dos nossos estudos, sugerem que a trealose pode tornar-se uma forte abordagem terapêutica para a MJD.

Palavras-chave:

Ataxia espinocerebelosa do tipo 3; Doença de Machado Joseph; Trealose; Chaperone químico; modelo MJD.

Chapter 1 – General Introduction

1. Polyglutamine diseases

The polyglutamine (polyQ) diseases are a set of inherited neurodegenerative disorders presenting an expanded stretch of consecutive CAG trinucleotides that encodes a glutamine tract in a widely expressed protein. These proteins are different for each disease, and vary both in size and localization of the glutamine segment. The variability in the size of the CAG repeats engenders a variety of phenotypes in each of these diseases. This group of diseases comprises Huntington's disease, dentalorubral-pallidoluysian atrophy (DRPLA), spinal bulbar muscular atrophy (SBMA), and the spinocerebellar ataxias (SCA) 1, 2, 3, 6, 7 and 17 (Havel *et al*., 2009; James *et al*., 2000,). The polyQ diseases are all autosomal dominant inherited except for the SBMA, which is X-linked (Fan *et al*., 2014).

Despite the variability within the polyQ diseases, they all present several common features: they are progressive, typically striking in midlife and cause neuronal dysfunction and eventual neuronal loss 10-20 years after the onset of the symptoms, along with physical and psychological complications (Zoghbi *et al*., 2000). Another common characteristic among polyQ disorders is the inverse relation between the size of the CAG repeat and the age of onset of the disease: the greater number of repeats leads to an earlier onset of symptoms (Dürr *et al*., 1996; Maciel *et al*., 1995; Matos *et al*., 2011; Ranum *et al*., 1995; Riess *et al*., 2008; Zoghbi *et al*., 2000).

Besides the polyQ tract, the proteins associated with each one of these disorders share no homology (Gatchel *et al*., 2005; Zoghbi *et al*., 2000), which suggests that the basis of the polyQ diseases is a toxic gain of function at the protein level, that increases along with the number of glutamine repeats (Paulson, 1999; Nagai *et al*., 2007).

1.1. Spinocerebellar ataxias

Spinocerebellar ataxias (SCAs) are a heterogeneous group of progressive neurodegenerative disorders, autosomal dominantly inherited, with a typical late-onset (usually between 30 and 50 years of age) and often fatal. These disorders are characterized by a degeneration of the cerebellum as well as changes in the brainstem and spinal tracts. SCA patients exhibit progressive gait and limb ataxia as the main clinical feature, variably associated with other symptoms that reflect damages beyond the cerebellum and its afferent and efferent pathways (Duenas *et al*., 2006; Paulson, 2007; Zoghbi, 2000).

2. Machado Joseph disease/Spinocerebellar ataxia type 3

Machado Joseph disease, also known as spinocerebellar ataxia type 3 (SCA3), is a polyQ disease with a CAG repeat expansion at the *MJD I/ATXN 3* gene, giving rise to a mutant protein – ataxin-3 - with a tract of glutamine repeats. It was first described in people of Portuguese Azorean descent, by Nakano *et al.* (1972), but it is now considered the most common form of dominantly inherited ataxia worldwide (Schöls *et al*., 2004). This disease was also identified in other countries such as Spain, Italy, Germany, China, Taiwan, Japan, Australia, Brazil, United States and Canada (Riess *et al*., 2008; Rosenberg, 1992; Sudarsky *et al*., 1995), geographically distributed with a great variability (Bettencourt *et al*., 2008).

2.1. Clinical features

MJD is essentially characterized by progressive gait and limb ataxia, also accompanied by signs and symptoms associated with damage in various other systems beyond the cerebellum, such as the pyramidal, extrapyramidal, motor neuron and oculomotor systems. Besides the cerebellar ataxia, other common features of MJD include progressive external ophtalmoplegia, dysarthria, dysphagia, pyramidal signs, dystonia, rigidity and distal muscle atrophies (Paulson, 2007; Paulson 2012; Lima and Coutinho, 1980). There are also evidences of non-motor symptoms associated with this disease, namely sleep disorders, cognitive and affective disturbances, psychiatric symptoms, olfactory dysfunction and peripheral neuropathy (Pedroso *et al*., 2013).

The age of onset of MJD is quite variable, although the symptoms usually start at 40 years old. The mean survival is 20 years after disease onset (Klockgether *et al*., 1998; Paulson, 2012).

2.2. Molecular and genetic features

2.2.1. The MJD I/ATXN3 gene

The *MJD I/ATXN3* gene was first tagged to the long arm of chromosome 14 (14q32.1), by Takiyama in 1993, and in the following year this same gene was cloned and it was shown that an unstable CAG repeat expansion motif was common to all MJD patients (Kawaguchi *et al*., 1994, Takiyama *et al*., 1993). The genomic structure of the MJD gene was determined and it was found to contain 11 exons and to span about 48 kb, with the CAG tract located

at the exon 10 (Ichikawa *et al*., 2001). Ichikawa et al. also reported that the MJD gene was ubiquitously transcribed into at least 4 different species of MJD transcripts, probably generated by alternative splicing of exons 10 and 11 and by alternative polyadenylation in exon 11 (Ichikawa *et al*., 2001).

The *MJD1/ATXN3* gene encodes for a protein – ataxin-3 – which contains a polyQ tract at the C-terminus encoded by the CAGn trinucleotide repeat. As CAG repeats are quite unstable, they range from 10 to 51 in healthy population, and they vary between 55 and 87 repeats in MJD patients (Bettencourt and Lima, 2011, Kawaguchi *et al*., 1994, Maciel *et al*., 2001, Nóbrega *et al*., 2012).

2.2.2. Ataxin-3 protein

Ataxin-3 was first described by Kawaguchi and colleagues in 1994 as an intracellular deubiquitinating enzyme who belongs to the group of cysteine proteases, and it presents a molecular weight of 42 kDa (Kawaguchi *et al*., 1994). This protein is distributed among different body tissues and cell types, despite the characteristic neuronal degeneration in MJD patients. It has been reported to exist predominantly in the cytoplasm, but also in the nucleus (where it accumulates when mutated) and in mitochondria (Paulson *et al*., 1997; Pozzi *et al*., 2008; Schmidt *et al*.; 1998, Trottier *et al*., 1998).

2.2.2.1. Structure and domains

Ataxin-3 is composed by a globular N-terminal domain (Josephine domain), with ubiquitin protease activity, combined with a flexible C-terminal tail that presents 2 or 3 ubiquitin-interacting motifs (UIMs) and the polyQ chain, whose length is variable, but whose expansion becomes associated with MJD over a certain threshold (Albrecht *et al*., 2004, Burnett *et al*., 2003, Masino *et al*., 2003).

Ataxin-3 is constituted by a highly conserved signal, upstream to the polyQ stretch, that determines the transportation into the nucleus – the putative nuclear localization signal (NLS). It was also characterized by the presence of 2 nuclear export signals (NES77 and NES141) that coincide with the Josephine domain. Moreover, another NES (NES174) was described and it is possible that can influence the nuclear export activity (Albrecht *et al*.,

2004, Antony *et al.*, 2009).

Figure 1 – Schematic representation of ataxin-3 protein structure.

Ataxin-3 is constituted by a conserved Josephine domain followed by a flexible C-terminal tail containing 2 or 3 UIM and a polyQ sequence of variable length. One functional NLS was described upstream of polyQ sequence and 3 NES were reported in this protein**. Abbreviations:** NES, nuclear export signal; NLS, nuclear localization signal; PolyQ, polyglutamine; UIMs, ubiquitin-interaction motifs.

2.2.2.2. Functions

The biological role of ataxin-3 is not yet fully elucidated, but it seems to participate in several cellular pathways. There is evidence suggesting the involvement of ataxin-3 in cellular protein quality control mechanisms, acting through the ubiquitin-proteasome system (UPS) (Doss-Pepe *et al.*, 2003), the main mechanism for the turnover of short-lived and damaged proteins in eukaryotic cells (Hershko and Ciechanover, 1998). Ataxin-3 contains ubiquitin interaction motifs (UIMs) that allow its connection with polyubiquitylated proteins (Burnett *et al.* 2003), and can operate as a de-ubiquitinating enzyme, editing the ubiquitin chain production and trimming these ubiquitinated species, regulating their ubiquitination status before their presentation to the proteasome (Paulson, 2012). After the interaction with ubiquitinated proteins, ataxin-3 interacts with other proteins, taking part in the substrate delivery to the proteasome (Doss-Pepe *et al.*, 2003).

Ataxin-3's role in the proteasome pathway was also supported by its interaction with VCP/p97, a key protein in the endoplasmic reticulum-associated degradation (ERAD) system. Ataxin-3 is involved in the extraction of misfolded proteins and unassembled polypeptides of protein complexes from the endoplasmic reticulum and in their delivery to the proteasomes (Boeddrich *et al.*, 2006; Doss-Pepe *et al.*, 2003; Hampton, 2002; Romisch, 2005; Wang *et al.*, 2006; Zhong and Pittman, 2006).

When the proteasomes are compromised, cells are forced to deal with misfolded proteins in other ways. Misfolded proteins are transported to a perinuclear location, near the microtubule-organizing centre (MTOC) to form aggresomes (Johnston *et al.*, 1998, García-Mata *et al.*, 1999). Endogenous ataxin-3 appears to be related with this process, since it co-localizes with both aggresomes and preaggresomes, and it collaborates with components of the complex that leads misfolded proteins to the MTOC (Burnett *et al.*,

2005). These structures are of extreme importance to the cellular homeostasis, since the defective proteins are then degraded by lysosomes (Markossian and Kurganov, 2004).

Endogenous ataxin-3 was recently shown to interact with two other constituents of the cytoskeleton – tubulin and microtubule-associated protein 2 (MAP2) (Mazzucchelli *et al*., 2009, Rodrigues *et al*., 2010) - and the importance of this interaction might be beyond the aggresome formation. The absence of endogenous ataxin-3 may engage in morphologic alterations and disorganization of several cytoskeleton constituents and also loss of cell adhesion and even cell death, which suggests a role of this protein in the organization of the cytoskeleton (Rodrigues *et al*., 2010).

A potential role of ataxin-3 in myogenesis was also suggested, related with integrins regulation, and affecting cytoskeleton organization and muscle differentiation (do Carmo Costa, 2010).

It was also described an involvement of endogenous ataxin-3 in transcription regulation, since it can bind target DNA and interact with transcription factors, both activators and repressors, influencing the regulation of expression of many genes (Li *et al*., 2002, Evert *et al*., 2003, Rodrigues *et al*., 2007). This relationship/connection might be related to its DUB activity, since ataxin-3 may interfere with the turnover of transcriptional regulators, thus interfering in the repressor complex formation and activity (Evert *et al*., 2006, Rodrigues *et al*., 2007).

2.3. Neuropathology

The neuropathological alterations in MJD involve neuronal loss in several brain regions such as the cerebellum, basal ganglia and brainstem (Rub *et al*., 2008). The neuropathology is characterized by degeneration and dysfunction of the cerebellum, cerebellar hemispheres and vermis (spinocerebellar pathways and dentate nucleus), brainstem (midbrain, pons and medulla), substantia nigra, striatum, thalamus, pontine nuclei, spinal cord and cranial nerves (including locus coeruleus and vestibular nuclei). Damages in visual, auditory, vestibular, somatosensory, ingestion, dopaminergic, cholinergic and urination-related systems were also observed. By comparison, structures such as cerebral and cerebellar cortexes, inferior olive and Purkinje cells are rather spared (Alves *et al*., 2008; Coutinho and Andrade, 1978; Dürr *et al*., 1996; Kanda *et al*., 1989; Rosenberg, 1992; Rub *et al*., 2008; Sudarsky and Coutinho, 1995), although in some cases, it has been reported a loss of granule and Purkinje cells in the

cerebellum, mainly in the vermis (Munoz *et al*., 2002). Most of the brains from MJD patients in advance disease stages weight less than those of healthy individuals (Iwabuchi *et al*., 1999, Rub *et al*., 2007). Recent studies using neuroimaging techniques namely magnetic resonance imaging (MRI) and quantitative 3-D volumetry confirmed a severe atrophy in the whole brainstem, whole cerebellum, cerebellar hemispheres and cerebellar vermis, putamen and caudate nuclei of MJD patients' brains (Schulz *et al*., 2010). A significant correlation of both brainstem and cerebellar atrophy with CAG repeat length, age, disease duration and degree of disability was also reported in MJD patients (Camargos *et al*., 2011).

Moreover, metabolic abnormalities leading to axonal dysfunction and glucose utilization deficits in cerebellum, brainstem and cerebral cortex were identified in MJD patients (D'Abreu *et al*., 2009, Soong and Liu, 1998).

The presence of neuronal intranuclear inclusion (NIIs) in both affected and non-afected areas of the brain is a hallmark of the pathology of MJD, although its cytotoxicity is still controversial. These nuclear inclusions are spherical and eosinophilic, non-membrane bound elements that contain a mixture of granular and filamentous structures, and their size varies between 0.7 and 3.7 uM. In their heterogeneous composition, besides normal and mutant ataxin-3 with expanded polyQ stretches, they include ubiquitin, molecular chaperones, proteasomal components and several transcription factors (Chai *et al*., 1999a; Chai *et al*., 1999b; Fujigasaki *et al*., 2000; Paulson *et al*., 1997; Schmidt *et al*., 1998, Schmidt *et al*., 2002,; Yamada *et al*., 2000; Yamada *et al*., 2002; Yamada *et al*., 2008).

Additionaly, extracellular accumulation and the presence of neuronal cytoplasmic inclusions (NCIs) with mutant ataxin-3 were described in MJD brains. The NCIs, fine granules with diameter of approximately 1.5 uM, present a similar distribution as the NIIs but exhibit an ubiquitin-negative profile (Hayashi *et al*., 2003; Yamada *et al*., 2002; Yamada *et al*., 2004; Yamada *et al*., 2008).

2.4. Pathogenesis

In spite of the well-described genetic basis of MJD, its molecular basis is still poorly understood. Nevertheless, many efforts were made to overcome this situation, and several cellular and molecular mechanisms have been proposed to explain the pathology of this disease.

In MJD, the conformational changes in ataxin-3 that result from the expanded polyQ tract, confer toxic properties to the protein, giving rise to altered molecular interactions that appear to trigger several pathogenic events, leading to neurodegeneration (Paulson *et al*., 1997).

The presence of neuronal inclusions containing the mutant protein is a common feature among the polyQ diseases, which suggests that misfolded proteins have pathological features (Ross, 1997). Although there is some controversy regarding this correlation (Michalik and Van Broeckhoven, 2003), the amount of neuronal inclusions was related to the number of CAG repeats and to the severity of the symptoms (Martindale *et al*., 1998), and it was proposed that they could originate an impairment in the axonal transport as well as in the nuclear function (Nóbrega and de Almeida, 2012; Paulson *et al*., 1997). As neuronal inclusions compromise the recruitment of several key proteins such as transcription factors, proteosomal components and chaperones, they affect various cellular pathways, with the depletion of important components (Chai *et al*., 1999a; Chai *et al*., 1999b; Paulson *et al*., 1997, Schmidt *et al*., 1998). It has also been observed a conformational change in polyQ monomers of ataxin-3, which acquire β-strand conformations (Nagai *et al*., 2007), and later assemble into oligomers (Bevivino and Loll, 2001; Takahashi *et al*., 2008). In MJD, both β-rich ataxin-3 monomers and oligomers can be toxic to cells (Bevivino and Loll, 2001; Nagai *et al*., 2007; Takahashi *et al*., 2008).

It has been suggested that the proteolytic cleavage of mutant ataxin-3 results in the formation of smaller, yet more toxic fragments, susceptible to aggregate and to generate cell dysfunction as their entry in the cell nucleus is facilitated (Goti *et al*., 2004; Haacke, *et al*., 2006, Simões *et al*., 2012; Simões *et al*., 2014).

As cells produce a large quantity of misfolded proteins, degradation systems like the UPS or autophagy are of extreme importance to control cellular function and maintain homeostasis (Nóbrega and de Almeida, 2012). Ataxin-3 was found to act as a polyubiquitin-biding protein as explained above, so a mutation in this protein can compromise this function and lead to the accumulation of misfolded proteins and consequently, to neurodegeneration and cell death (Li and Chin, 2007).

There are evidences indicating that macroautophagy plays an important role in the degradation of insoluble aggregate-prone proteins that are inefficiently degraded by the UPS and is an essential mechanism for neuronal survival (Cuervo, 2004). An impairment in this pathway has been described in MJD models: it was shown that mutant ataxin-3 inclusions sequestered important autophagy proteins and accumulated in MJD patients' brains (Nascimento-Ferreira *et al*., 2011). Inefficiency by these cellular quality control systems

induced by mutant ataxin-3 can enhance neurodegeneration and lead to cell death (Nóbrega and de Almeida, 2012).

Transcriptional deregulation, mitochondrial dysfunction and oxidative stress, and deregulation of calcium homeostasis may also be probable toxic mechanisms by which the misfolded ataxin-3 originates cytotoxicity (Costa and Paulson, 2012, Nóbrega and de Almeida, 2012, Bauer and Nukina, 2009).

Neuroinflammation is also shown to be another important mechanism of pathogenesis in MJD, with the upregulation of some inflammatory genes, although it seems to be activated only in later stages of the disease (Evert *et al.*, 2001, Evert *et al.*, 2003).

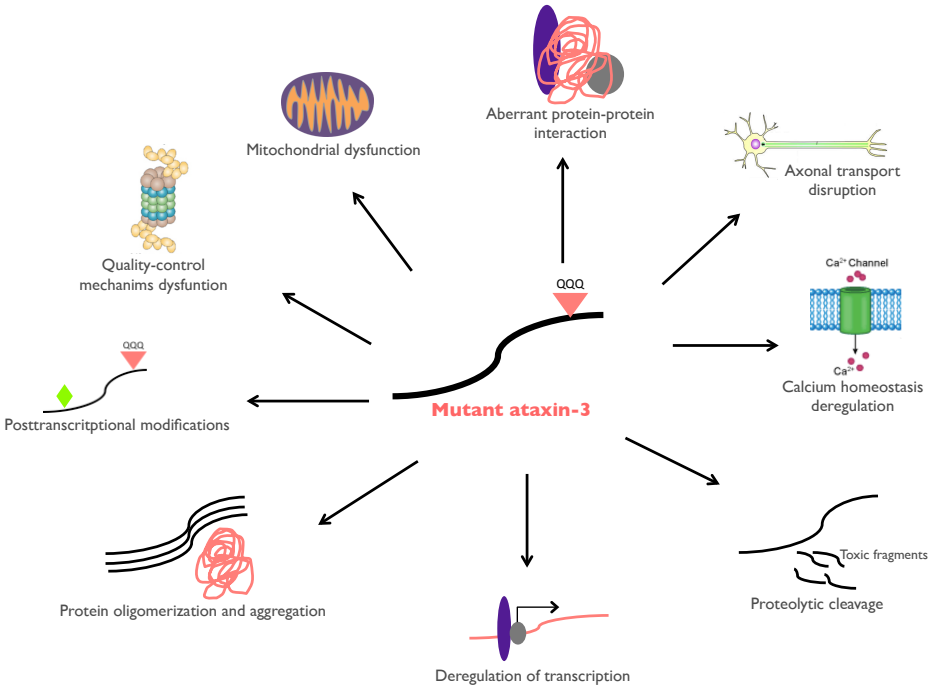


Figure 2 – Mechanisms of pathogenesis in Machado-Joseph disease. (Adapted from Nóbrega and de Almeida, 2012).

Several mechanisms can contribute to the pathogenesis in MJD. The presence of mutant ataxin-3 in the cellular environment triggers several events that lead to neurodegeneration. The expanded polyQ tract in ataxin-3 confers toxicity to this protein, and can generate oligomerization and aggregation, formation of toxic fragments or posttranscriptional modifications, important to the neuronal dysfunction and cytotoxicity present in this disease. Regarding the participation of ataxin-3 in the cell UPS, a mutation in this protein can compromise its normal function with an impairment of this system and thus contributing to the dysfunction of cellular quality-control mechanisms. Other mechanisms such as transcriptional deregulation, mitochondrial dysfunction, aberrant protein-protein interactions, axonal transport disruption and calcium homeostasis deregulation can also be important to the pathogenesis of MJD.

2.5. Animal models of MJD

Animal models of diseases are of great utility regarding the understanding of the pathologies and the development of therapies. Since no model animal is known to naturally develop MJD's characteristic phenotype, the models developed until now result from the breeding of transgenic lineages expressing human full-length polyQ-expanded ataxin-3 or a truncated form of this protein (Matos *et al*., 2011). The animals currently used for these models are mice, rats, fruit flies and worms (Costa and Paulson, 2012).

The use of invertebrates like *Drosophila* and *C. elegans* as models of disease allowed the understanding of several aspects related to the pathogenesis of MJD, such as aggregation, proteolysis and toxicity of mutant ataxin-3, and helped to clarify the role of ataxin-3 (Costa and Paulson, 2012). However, rodent models are preferred, as they share crucial similarities with humans, at molecular, anatomical and physiological level (Costa and Paulson, 2012).

The first mouse model of MJD was developed in 1996 by Ikeda and colleagues. In this study it was expressed full length expanded ataxin-3 (with 79Q) in one transgenic line and a truncated transcript in another, both selectively expressed in Purkinje cells, and it was demonstrated the potential involvement of the expanded polyQ tract in the development of the disease (Ikeda *et al*., 1996). This model presented limitations: the expression of full-length ataxin-3 complementary DNA did not lead to pathologic changes whereas the expression of a truncated form of this protein originated an early and severe phenotype, along with a marked degeneration of the cerebellum (Costa and Paulson, 2012; Matos *et al*., 2011).

In 2002, Cemal *et al*. (2002) developed a mouse model created by genomic integration of the whole human expanded ATXN3 gene and respective regulatory elements in a yeast artificial chromosome (Matos *et al*., 2011; Costa and Paulson, 2012). The transgenic human ataxin-3 was expressed at physiological levels in the same tissue as endogenous murine ataxin-3, and both neurodegeneration and neuronal intranuclear inclusions developed in subpopulations usually affected in MJD patients. At 5 months of age, these mice showed a mild and slowly progressive motor phenotype (Cemal *et al*., 2002).

Another MJD model was generated injecting lentivirus expressing human ATXN3 in rat brains, reproducing several features of MJD neuropathology and serving as a useful tool to the study of this disease (Alves *et al*., 2008; Alves *et al*., 2010).

Several other mouse models were created using complementary DNA encoding a particular isoform of ATXN3 driven by a foreign promoter, and as they result from DNA random integration, they differ in the copy number of integrated transgenes (see Costa and

Paulson, 2012 and Matos *et al*., 2011 for a review). In mouse models, the severity of the pathology and symptoms is usually proportional to the expression level of the mutant protein, resembling MJD patients (Costa and Paulson, 2012).

A mouse model developed by Torashima in 2008 has been widely studied and characterized by Luis Almeida's group (Duarte-Neves *et al*, 2015; Mendonça *et al*., 2015; Nascimento-Ferreira *et al*., 2013; Nóbrega *et al*., 2013;; Simões *et al*., 2012). This model was generated in a C57/BL6 background, expressing an expanded polyglutamine protein in Purkinje cells, using a complementary DNA encoding a truncated form of human ataxin-3 with 69 glutamine repeats, driven by a Purkinje cell-specific L7 promoter (Torashima *et al*., 2008). These mice showed severe ataxic behaviour since the 3rd week of age, with poor balance and rotarod performance as expected, since Purkinje cells play a critical role in the regulation of motor coordination (Torashima *et al*., 2008). They also presented a characteristic MJD gait, with irregularly shorter strides and wider widths between right and left limbs (Torashima *et al*., 2008; Nascimento-Ferreira *et al*., 2013).

Regarding neuropathological features, they presented cerebellar degeneration along with decreased Purkinje cells arborisation and a disarrangement of the Purkinke cell layer (Oue *et al*., 2009). Neuronal intranuclear inclusions enriched with mutant ataxin-3 were found in the cerebellar cortex and in axon terminals of Purkinje cells in the deep cerebellar nuclei since 40 days of age (Torashima *et al*., 2008). Moreover, severe alterations in synaptic transmission and in action potential generation related to Purkinje cells were observed in this mouse model (Oue *et al*., 2009). These features make this model time and cost-effective, mimicking the late stage of MJD (Nóbrega *et al*., 2013).

Model	Transgene/promoter	Ataxin-3 distribution	Neuronal inclusions	Pathology	Reference
Mouse	MJD1a cDNA fragment with 79 CAGs under L7 promoter	Purkinje cells	No description	Cerebellar atrophy, ataxic phenotype, wide gait	(Ikeda et al., 1996)
	Full-length MJD1 YAC with 76 or 84 CAGs under the control of its own regulatory elements	ubiquitous	Ubiquitin-positive NIIs predominantly found in pontine and dentate neurons	Cell loss in pons and cerebellum, peripheral neuropathy, ataxic phenotype, tremor, wide gait, lowered pelvis, reduced motor and exploratory behaviour	(Cemal et al., 2002)
	Full-length MJD1a cDNA with 71 CAGs under mouse prion promoter	Brain and spinal cord	NIIs in deep cerebellar and pontine nuclei, substantia nigra and spinal cord	Progressive postural instability, gait and limb ataxia, weight loss, premature death	(Goti et al., 2004)
	Full-length human ataxin-3 cDNA with 70 or 148 CAGs under control of the murine prion promoter	Several brain regions including cortex, hippocampus, pons and cerebellum	Ubiquitin-positive NIIs in cortex, hippocampus, pons, cerebellum and cerebellar nuclei	Degeneration of Purkinje cells, reduced turnover of DA and 5-HT, tremor, wide gait, reduced activity, premature death	(Bichelmeier et al., 2007)
	HA-tagged full-length human MJD1a cDNA with 79 CAGs under mouse prion promoter	Several brain regions including cerebellum, pontine neurons and substantia nigra	NIIs in neurons of dentate nucleus, pontine neurons and substantia nigra	Ataxic phenotype, impaired motor coordination, ataxic gait, abnormal posture, weight loss	(Chou et al., 2008)
	HA-tagged human ataxin-3 fragment cDNA with 69 CAGs under L7 promoter	Purkinje cells and deep cerebellar nuclei	Ubiquitin-positive NIIs periplasmic in Purkinje cells	Cerebellar atrophy, ataxic phenotype, impairment of dendritic differentiation	(Oue et al., 2009; Torashima et al., 2008)
	Full-length human ataxin-3c cDNA with 77 CAGs using the Tet-Off system under the control of a hamster prion promoter	Brain, with stronger immunoreactivity in cerebellum, predominantly in glial cells	NIIs in some neuronal cells of the cerebellar cortex	Degeneration of Purkinje cells and of the molecular layer of the cerebellum, hyperactivity, motor deficits, gait disturbance	(Boy et al., 2009)
	Full-length human ataxin-3c cDNA with 148 CAGs under the rat huntingtin promoter	Ubiquitous in brain	NIIs in the red nucleus, pons and cerebellum (including Purkinje cells)	Degeneration of Purkinje cells, impaired motor coordination, hyperactivity, reduced motor learning	(Boy et al.,, 2010)
	Full-length human ataxin-3c cDNA with 94 CAGs under the cytomegalovirus (CMV) promoter	ubiquitous	No proper NIIs observed	Neuronal atrophy, astrogliosis, impaired motor coordination, reduced locomotor activity	(Silva-Fernandes et al., 2010)

Model	Transgene/promoter	Ataxin-3 distribution	Neuronal inclusions	Pathology	Reference
Mouse	Truncated N-terminal ataxin-3 cDNA	Brain and spinal cord	Neuronal cytoplasmic inclusions	Altered endoplasmic reticulum-mediated response, neuronal death, tremor, gaita taxia, weight loss, premature death	(Hubener et al., 2011)
Rat	Myc tagged full-length human MJD1a cDNA with 72 CAGs under the phosphoglycerate kinase 1 (PGK) promoter	Substantia nigra or striatum (depending on the site of injection)	Ubiquitin-positive NIIs in the substantia nigra or striatum (depending on the site of injection)	In substantia nigra, loss of dopaminergic markers; in striatum, loss of neuronal markers	(Alves et al., 2008)
Drosophila melanogaster	HA tagged human MJD1 cDNA fragment with 78 CAGs using a glass gene promoter or elav gene promoter	Glass: eye cells; elav: peripheral and central nervous system	Glass: NIIs in eye cells; elav: NIIs in eye cells and brain	Glass: late onset eye degeneration; elav: early death with loss of integrity of the nervous system	(Warrick et al., 1998)
	HA or myc tagged full-length ataxin-3 with 78 or 84 CAGs, respectively, using a glass gene promoter or elav gene promoter	Glass: eye cells; elav: peripheral and central nervous system	Glass: NIIs in eye cells; elav: NIIs in eye cells and brain	Glass: severe and progressive adult-onset degeneration; elav: tremor and early death	(Warrick et al., 2005)
Caenorhabditis elegans	Full-length and truncated MJD1 cDNA with 91 or 130 CAGs and 63 or 127 CAGs, respectively, under the control of a pan neuronal promoter	ubiquitous	Aggregates may be present in the cytoplasm, essentially in the perinuclear region and rarely in the nucleus	Neuronal dysfunction, interruption of synaptic transmission, UPS impairment, locomotor abnormalities	(Khan et al., 2006)
	Full-length ataxin-3 cDNA with 130 CAGs and truncated ataxin-3 with 75 or 128 CAGs under the control of a pan neuronal promoter	Through the nervous system	Foci formation detected in some neurons in both nucleus and cytoplasm	Motor dysfunction, lethargy, small reduction of life span	(Teixeira-Castro et al., 2011)

Table 1 – Animal models of Machado Joseph disease (Adapted from Matos et al., 2011).

3. Trehalose

Trehalose, also named α- D-glucopyranosyl α- D-glucopyranoside, is a sugar contained in nearly 100 species of plants, algae, fungi, yeasts, bacteria, insects and other invertebrates (Elbein, 1974). The role of trehalose in these species varies widely. Among other roles, trehalose enhances desiccation tolerance of several plants, which withstand temperatures up to 100°C; allows the prolonged survival of a variety of products in the dried state; provides cold protection in several organisms; acts as an energy source in lower organisms, (Adams *et al*., 1990; Elbein, 1974; McBride and Ensign, 1987; Newman *et al*., 1993; Roser, 1991; Singer *et al*., 1998a; Van Dijck *et al*., 1995).

In spite of the wide variety of organism from which one can extract trehalose, this was considered a rare sugar. Over the years, synthesis methods were developed to allow a large-scale production of this sugar, and nowadays its use is well described in the food, cosmetic and pharmaceutical industry (Ohtake and Wang, 2011).

3.1. Physical and chemical properties

Trehalose is a nonreducing sugar that presents remarkable stability (Ohtake and Wang, 2011) as the two glucopyranose rings of this disaccharide are linked through the reducing end of the glycosyl residues (α-carbons).

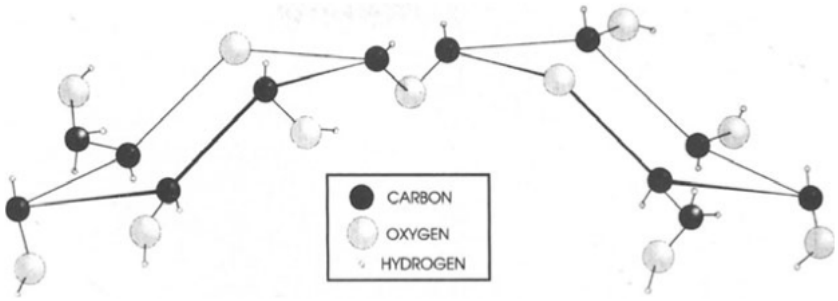


Figure 3 – Trehalose, α-D-glucopyranosyl α-D-glucopyranoside. (Adapted from Colaço and Roser, 1995).

Trehalose is usually found in the dehydrated form and presents low hygroscopicity (Ohtake and Wang, 2011). Trehalose is soluble in water (34g/100g H2O at 5°C and 40.6-69g/100g H2O at 20°C) and has a great stability to hydrolysis due to the low energy of its glycosidic bond (Colaco and Roser, 1995; Kubota, 2008; Miller *et al*., 1997; Roser, 1991). The rate of hydrolysis is very important for the stability of labile biological, as reducing

monosaccharides (like glucose) can easily undergo Maillard reaction, with a fall in pH, which is a severe problem among food processing (Colaço and Roser, 1995; Ohtake and Wang, 2011).

The physical properties of trehalose can also derive from its flexibility or conformation, as these features are influenced by the intramolecular hydrogen bonds between the monosaccharide residues. No direct intramolecular hydrogen bonds have been reported for trehalose in the crystalline form (Brown *et al*., 1972). Trehalose presents a high number of equatorial –OH groups, interacting strongly with water in solution, and being easily included in the water cluster (Tanaka, 2009; Ohtake and Wang, 2011). Altogether, these unique physical properties are responsible for trehalose's stabilizing effects.

Table 2 – Properties of trehalose. (Adapted from Higashiyama, 2002).

Melting point	Dihydrate	97.0°C
	anhydride	210.5°C
Heat of fusion	dihydrate	57.8 kJ mol^{-1}
	anhydride	53.4 kJ mol^{-1}
Solubility	68.9g/100g H_2O at 20°C	
Relative sweetness	45% of sucrose	
Digestibility	Digested and absorbed by the small intestine	
pH stability of solution	>99% (pH 3.5-10, at 100°C for 24h)	
Heat stability of solution	>99% (at 120°C for 90 min)	

3.2. Stabilization properties

Trehalose is known for its ability to stabilize lipids and proteins, and complex biologicals such as viruses, bacteria and tissues, and this property is likely related to its molecular conformation (Richards *et al*., 2002). Several theories have been suggested to explain the mechanisms by which trehalose stabilizes biological molecules. Here we revise three main theories.

Colaço and Roser (1995) arranged the possible mechanisms by which trehalose stabilizes biologic molecules in three categories: 1) water replacement; 2) glass transformation and 3) chemical stability, but these mechanisms are not mutually exclusive and may all contribute to the stabilizing effects of this disaccharide (Richards *et al*., 2002). The water replacement hypothesis suggests that biologic macromolecules are commonly stabilized by water that forms hydrogen bonds around these molecules. The great flexibility of the glycosidic bond

between the two D-glucose molecules of this disaccharide may allow its coordination with the irregular polar groups of macromolecules (Colaço and Roser, 1995; Richards *et al*., 2002). The glass transformation theory is based on the ability of sugars to solidify from solution as glass instead of crystallizing. As trehalose forms a non-hygroscopic glass stable at high temperatures it stabilizes biomolecules in a way that allows them to return to their native structure and function when rehydrated (Colaço and Roser, 1995; Crowe and Crowe, 2000; Richards *et al*., 2002). The chemical stability theory results from the unique chemical properties of trehalose, described above, that confer its stabilization effect.

Lins and collaborators proposed three major hypothesis for the mechanism by which trehalose stabilizes macromolecules: 1) direct interaction between trehalose and the macromolecule structure through hydrogen bonds (water replacement hypothesis); 2) trehalose traps the water molecules close to the surface of the protein (water-layer hypothesis) and 3) trehalose forms a viscous matrix around the protein surface (mechanical-entrapment hypothesis) (Lins *et al*., 2004). These three hypotheses share the reduction, by trehalose, of interactions between hydrophobic regions on the surface of mutant proteins and water molecules. The prevention of hydrophobic interactions between mutant proteins leads to an impediment on the formation of aggregates and oligomers (Seki *et al*., 2010).

Ohtake and Wang (2011) described a direct and an indirect mechanism responsible for these stabilization properties. The direct mechanism assumes a "direct interaction" of trehalose with the compound that is being stabilized, both in the dry state (through hydrogen bonding with proteins) and in solution (through interaction with metal ions to stabilize vitamins). The "indirect interaction" can be described as an effect of this sugar in the surrounding environment of the compound that is being stabilized, resulting in a restriction of mobility and increased hydration (Ohtake and Wang, 2011).

Overall, the unique physical and chemical properties of trehalose seem to grant it the capacity to interact with a molecule and/or its surrounding environment, reducing hydrophobic interactions and avoiding undesirable following events so that stabilization can be achieved.

3.3. Biological properties

The correct folding of proteins into their functional compact native structures is the most crucial and universal feature of biological self-assembly (Dobson, 2003) and a failure in this process will result in the malfunction of living systems and thus in diseases (Thomas *et*

al., 1995). Incompletely folded proteins inevitably expose some hydrophobic regions of their structure that otherwise would be hidden in the native state, so they have a bigger tendency to interact inappropriately with other molecules within the cellular milieu (Ellis, 2001). This interaction must then be avoided and living organisms display strategies to prevent this situation, as the presence of a great variety of molecular chaperones in all types of cells and cellular compartments. A clear evidence that molecular chaperones are of extreme importance in the prevention of misfolding of proteins is the increase of these species during cellular stress, like heat shock (Bukau and Horwich, 1998; Dobson, 2003). Besides protecting proteins during its folding, some molecular chaperones rescue misfolded and aggregated proteins and give them a second opportunity to fold correctly (Bukau and Horwich, 1998).

Several studies indicate that trehalose, along with other small stress molecules, act as chemical chaperones, and have the ability to stabilize proteins, protecting them from denaturation and aggregation and thus maintaining its normal functions under extreme environmental stresses (Singer and Lindquist, 1998; Santos and da Costa, 2002; Borges *et al*., 2002).

Moreover, trehalose has been shown to induce autophagy. It was first reported in 2007 by Sarkar and colleagues that trehalose induces mTOR-independent autophagy and promotes the degradation of diverse aggregate-prone proteins in cell culture models (Sarkar *et al*., 2007). Trehalose induced the clearance of autophagy substrates like mutant huntingtin and mutant species of a-synuclein, in *in vitro* models of Huntington and Parkinson disease, respectively (Sarkar *et al*., 2007). Sarkar et al. also described that as an autophagy inducer, trehalose also protects cells against following pro-apoptotic insults through the mitochondrial pathway (Sarkar *et al*., 2007).

The ability of trehalose to induce autophagy is of great interest since it has been suggested that autophagy plays a crucial role in neurodegenerative disorders, including Parkinson and Alzheimer diseases and also polyQ diseases, like MJD (Berger *et al*., 2006; Mizushima and Hara, 2006; Nascimento-Ferreira *et al*., 2011; Nixon *et al*., 2005; Qin *et al*., 2003; Ravikumar *et al*., 2002; Rubinsztein *et al*., 2005; Rubinsztein *et al*., 2006; Webb *et al*., 2003; 2013; Williams *et al*., 2006) (See section 3.4.2).

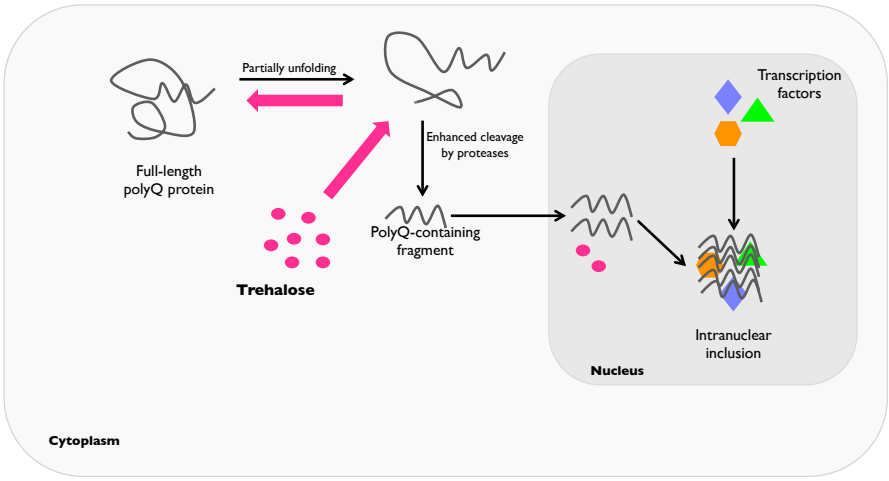


Figure 4 – A possible strategy for the treatment of polyglutamine diseases with trehalose as a chemical chaperone. (Adapted from Tanaka *et al*., 2005)

Trehalose stabilizes aggregation-prone polyglutamine (polyQ) containing proteins and inhibits their aggregation (pink arrows). This increase in stability could also enhance the resistance of polyQ-containing proteins to proteolysis and prevent subsequent translocation of cleaved fragments containing expanded polyglutamines to the nucleus, where the truncated proteins interact with several transcription factors and encourage cellular dysfunction.

3.4. Trehalose applications

3.4.1. Trehalose use as a stabilizer in food, cosmetics and pharmaceutical industry

Trehalose is mainly used as a stabilizer in food industry and cosmetics, and also in biotechnology and pharmaceutical industry since it is safe and its properties grant this function (Richards *et al*., 2002). In 2000, trehalose was considered safe (generally regarded as safe) by the US Food and Drug Administration and, in Europe, its regulatory approval as a food ingredient was assumed in 2001 (Ohtake and Wang, 2011).

Trehalose prevents starch retrogradation, protein denaturation and lipid degradation and shows poor reactivity against amino compounds in food. It also masks unpleasant odors and tastes (Higashiyama, 2002). Trehalose is used in several commercialized pharmaceutical products, but its use is still limited, as compared with other saccharides like mannitol or sucrose. Nevertheless, several products containing trehalose are presently in development. There are several reasons for including trehalose in pharmaceutical formulations, related with the stabilization properties of this sugar, as described above. There are also studies taking place in which this disaccharide is being evaluated as the key component of the formulation (Ohtake and Wang, 2011).

3.4.2. Trehalose use as a drug for protein-misfolding diseases

The stabilization of proteins that aggregate and lead to disease is already considered an effective therapeutic strategy (Miroy *et al*., 1996). In studies of transthyretin amyloid disease, trehalose, among other small molecules, was found to stabilize the native tetrameric state of a potentially amyloidogenic human protein, leading to an increase of the kinetic barrier associated with the misfolding (Hammarstrom *et al*., 2002; Hammarstrom *et al*., 2003) and these results show the importance of protein misfolding energetics in the pathogenesis (Cohen and Kelly, 2003; Dobson, 2003).

Concerning the results of the studies using small stress molecules to stabilize amyloidogenic proteins, Arora and colleagues reported the inhibition of insulin amyloid formation *in vitro* by trehalose (Arora *et al*., 2004). Being the abnormal accumulation of amyloid oligomers, protofibrils or fibrils one major factor contributing to neurodegeneration, the use of trehalose was considered of great interest in the treatment of diseases such as Alzheimer's, Parkinson's, among others (Arora *et al*., 2004). In fact, the beneficial effects of trehalose were already described in AD pathology, inhibiting aggregation of Aβ and reducing its cytotoxicity *in vitro* (Liu *et al*., 2005) and in transgenic mouse models of AD (Perucho *et al*., 2012; Du *et al*., 2013), as well as in Parkinson's disease, in which this disaccharide improved several pathological features of a mouse model (Sarkar *et al*., 2014).

The therapeutic interest of trehalose, regarding its function as a protein stabilizer, has been reported in several other cases. It decreases the size of *de novo* produced PrP^{Sc} aggregates and alters their localization in the cell, thus significantly protecting prion-infected cells from being damaged by oxidative stress (Béranger *et al*., 2008).

Trehalose was found to have beneficial effects in OPMD, reducing the aggregation and toxicity of the mutant poly(A) binding protein nuclear 1 (PABPN1) in cell models of the disease and, furthermore, reducing the aggregation and decreasing the number of TUNEL-labelled nuclei in skeletal muscle of a mouse model of the same disorder, along with the attenuation of muscle weakness (Davies *et al*., 2005). Recently, it was reported to prolong life span of a mouse model of amyotrophic lateral sclerosis, to attenuate the progression of disease signs, and to decrease the accumulation of SOD1 aggregates and enhance motor neuron survival (Castillo *et al*., 2013; Zhang *et al*., 2014).

Aguib and collaborators reported that the treatment with trehalose resulted in induction of autophagy together with reduction of PrP^{Sc}, both *in vitro* and *in vivo*, ameliorating prion

diseases (Aguib *et al*., 2009). Castillo and colleagues associated the protective effects of trehalose in disease models of ALS with the upregulation of several essential autophagy-regulatory genes and with an increased translocation to the nucleus of an important transcription factor, forkhead box O1, involved in the regulation of this pathway among neurons (Castillo *et al*., 2013). Zhang and collaborators confirmed the beneficial effects of trehalose in a mouse model of ALS, delaying disease onset and progression and reducing motor neuron loss in their spinal cord and also decreasing SOD1 and SQSTM1/p62 aggregation, reducing accumulation of ubiquitinated proteins and improving the autophagic flux (Zhang *et al*., 2014). Trehalose was also found to protect mitochondria, inhibit the proapoptotic pathway and reduce skeketal muscle denervation in this mouse model of ALS (Zhang *et al*., 2014).

It was described a function for trehalose as a protector against induced oxidative damage in prion-infected mammalian cells (Béranger *et al*., 2008). This role was first reported in yeast and it was proposed that trehalose inhibited the covalent modifications of proteins by oxygen radicals (Benaroudj *et al*., 2001).

Recently, regarding the studies of Minutoli *et al.* in endotoxic shock (2008), Sarkar and collaborators proposed for trehalose a role as a protector against neuroinflammation (Sarkar *et al*., 2014). In a mouse model of Parkinson disease, trehalose reduced microglial activation and astrocytic hypertrophy, consistent with the sparing of motor deficits characteristic of these mice (Sarkar *et al*., 2014).

Overall, numerous *in vitro* and *in vivo* studies have demonstrated the benefits of trehalose in various protein-misfolding diseases. Besides its ability to prevent protein misfolding, trehalose can enhance mTOR-independent autophagy and also exhibit antioxidant and anti-inflammatory effects.

Table 3 – *In vitro* and *in vivo* studies of trehalose in protein-misfolding diseases.

Neurodegenerative disease	*In vitro* studies	*In vivo* studies	References
Alzheimer's disease	Inhibition of Aβ aggregation, reduction of cytotoxicity	Reduction of Aβ and tau aggregates, prevention of apoptosis in hippocampus and cerebral cortex, , increased conversion of the autophagic marker LC3-I to LC3-II, improvement of cognitive and learning ability	(Liu *et al*., 2005; Perucho *et al*., 2012; Du *et al*., 2013; Schaeffer *et al*., 2012)
Amyotrophic lateral sclerosis (ALS)	Increased degradation of mutant SOD1 by autophagy and protection against cytotoxicity	Reduced accumulation of SOD1 aggregates, upregulation of key autophagy-related genes and prolonged life span	(Castillo *et al*., 2013)
Huntington disease	Inhibition of mutant huntingtin aggregates formation and increased cell viability	Reduced weight loss of Tg mice, reduced brain atrophy, increased brain weight, decreased ubiquitin-	(Tanaka *et al*., 2004)

		positive intranuclear huntingtin aggregates in motor cortex, striatum and liver, improved motor dysfunction and extended lifespan	
Oculopharyngeal muscular dystrophy (OPMD)	Reduction of PABPN1 aggregation and toxicity	Decrease in PABPN1 aggregation, attenuation of phenotype, delay of disease onset	(Davies *et al*., 2006; Wada *et al*., 2014)
Parkinson disease	Reduced aggregation and cell death, reduced microglial activation and astrocytic hypertrophy, protection against neuroinflammation, stimulation of autophagy in an mTOR-independent manner and protection against secondary apoptotic effects	Reduction of α-synuclein aggregates and activation of the autophagy pathway	(Lan *et al*., 2012; Sarkar *et al*., 2007, 2014; Yu *et al*., 2012)
Prion diseases	Decreasing size of *de novo* produced PrP^{Sc} aggregates and modification of their subcellular localization, protection from induced oxidative damage, induction of autophagy	Delayed accumulation of PrP^{Sc} in the spleen of prion-infected mice	(Béranger *et al*., 2008; Aguib *et al*., 2009)
Spinocerebellar ataxia type 14	Inhibition of mutant kinase Cγ aggregation, reduction of cytotoxicity, alleviation of improper development of dendrites in Purkinje cells without aggregates	-	(Seki *et al*., 2010)
Spinocerebellar ataxia type 17	Reduced aggregation	Amelioration of motor coordination and gait impairment, increased cerebellum weight and amelioration of cerebellar atrophy, reduced hyperactivity, attenuation of astrogliosis and neurodegeneration	(Chen *et al*., 2015)
Transthyretin amyloid disease	Inhibition of amyloid fibril formation, probable increase of the kinetic barrier associated with misfolding	-	(Hammarstrom *et al*., 2001; Hammarstrom *et al*., 2003)

3.4.2.1. Trehalose application in alleviating polyglutamine-mediated pathology

Polyglutamine diseases, as described above, are autosomal dominant inherited diseases that present expanded polyglutamine tracts in a widely expressed protein. One of the hallmarks of these disorders is the formation of intranuclear inclusions of polyglutamine-containing proteins in the brain and, although the relation between the polyQ aggregation and cellular toxicity is quite controversial, this production of aggregates containing mutant proteins can be related to cellular dysfunction. The inhibition of polyQ aggregation can, this way, be an option in the treatment of these diseases.

The therapeutic potential of small molecules in inhibiting the formation of polyQ aggregates has been widely accepted (Hughes and Olson, 2001; Heiser *et al*., 2000; Heiser *et al*., 2002) and in 2004, Tanaka *et al*. developed an *in vitro* model for polyQ disorders and screened several of these molecules (nontoxic, safe to administer and to be orally administered) to assess a possible beneficial effect in polyQ diseases (Tanaka *et al*., 2004). Among these small molecules, trehalose was the most effective in reducing the aggregation of mutant protein, and this result was also proved in a mouse model of Huntington's disease, with a reduction of polyQ aggregates in the brain and liver, an improvement of the associated motor dysfunction and an extended life span of the treated animals (Tanaka *et al*., 2004).

In 2010, Seki and colleagues reported that trehalose could inhibit (in neuronal cell lines and in cultured Purkinje cells) the aggregation of the mutant protein involved in SCA14 (protein kinase Cγ) and its cytotoxic effects (Seki *et al*., 2010). More recently, Chen and colleagues described a beneficial effect of trehalose in SCA17 pathological features both *in vitro* and *in vivo* (Chen *et al*., 2015).

In fact, regarding MJD, it was already established a beneficial relationship between the reduction of mutant ataxin-3 aggregates and the improvement of neuropathology and alleviation of the characteristic motor impairments of the disease (Nascimento-Ferreira, *et al*., 2011; Nóbrega, *et al*., 2014).

Although it has been proven that trehalose can be a promising therapeutic approach to several disorders, including polyQ diseases, studies of the effects of this disaccharide in MJD are still lacking. Research in human patients do not permit the evaluation of the neuropathology before and after the treatment, so it would be of great importance to study the effect of this drug in cellular and animal models of MJD. In the present study, we used a MJD transgenic mouse model to investigate whether trehalose can alleviate both motor behaviour and neurodegeneration characteristic of this disorder. Thereby, the purpose of our study was to investigate the *in vivo* relevance of trehalose as a therapy for MJD.

Objectives:

In the present study, we aim at investigating whether trehalose can alleviate both motor behaviour and neurodegeneration characteristic of this disorder in a MJD transgenic mouse model.

For this purpose we treated MJD transgenic mice (Torashima *et al*., 2008) with 2% trehalose for 30 weeks and investigated whether trehalose can alleviate MJD motor behaviour and rescue the neuropathology in this mouse model of MJD.

We expect that this study will establish *in vivo* the relevance of trehalose as a therapy for MJD.

Chapter 2 – Materials and Methods

2. Materials and methods

2.1 *In vivo* experiments

2.1.1. Transgenic mouse model

A MJD mouse model (C57BL/6 background) generated by expressing the N-terminal truncated human ataxin-3 with 69 glutamine repeats (69Q) and an N-terminal hemaglutinin (HA) epitope driven specifically in cerebellar Purkinje cells by the L7 promoter was used (Torashima *et al*., 2008). The colony was maintained at the animal house facility of the Centre for Neuroscience and Cell Biology (CNC) of the University of Coimbra by backcrossing heterozygous males with C57BL/6 females (Nascimento-Ferreira *et al*., 2013).

The animals were weaned and genotyped at 4 weeks old. Animals were housed in groups (2-5 per cage) in plastic cages (365 x 207 x 140 mm), in a temperature-controlled room and maintained on a 12 hours light/dark cycle at a room with constant temperature (22 ± 2°C) and humidity (55 ± 15%). Food and water (or trehalose aqueous solution) were available *ad libitum*. The animals were allowed one week of acclimation to the surroundings before the beginning of the behavioral tests. The experiments were carried out in accordance with the European Community Council Directive (86/609/EEC) for the care and use of laboratory animals. Genotype was confirmed by PCR.

2.1.2. Experimental groups and trehalose treatment

Animals from different progenitors were randomly distributed into treatment and control groups (12 animals/group; 7 females and 5 males) that received either 2% trehalose in drinking water or fresh distilled water, respectively, from 5 to 35 weeks of age. A fresh solution of 2% trehalose in water was prepared and changed twice a week until euthanasia of the animals. Physical state of animals was evaluated daily and weight measured every week. Study was conducted until significant improvements in behaviour were observed in the Treatment Group *vs* Control Group and were maintained for several weeks (Fig. 5).

2.1.3 Behavioural assessment

Mice were tested for behavioural background at 5 weeks of age (before the beginning of trehalose treatment, t=0). Behaviour was then evaluated at different time points (t=2, t=4, t=6, t=8, t=11, t=14, t=17, t=20, t=24 and t=28 weeks of treatment as shown in figure 5) by an experienced operator in a blind fashion way. All tests were performed in the same dark room after at least 60 minutes of acclimatization.

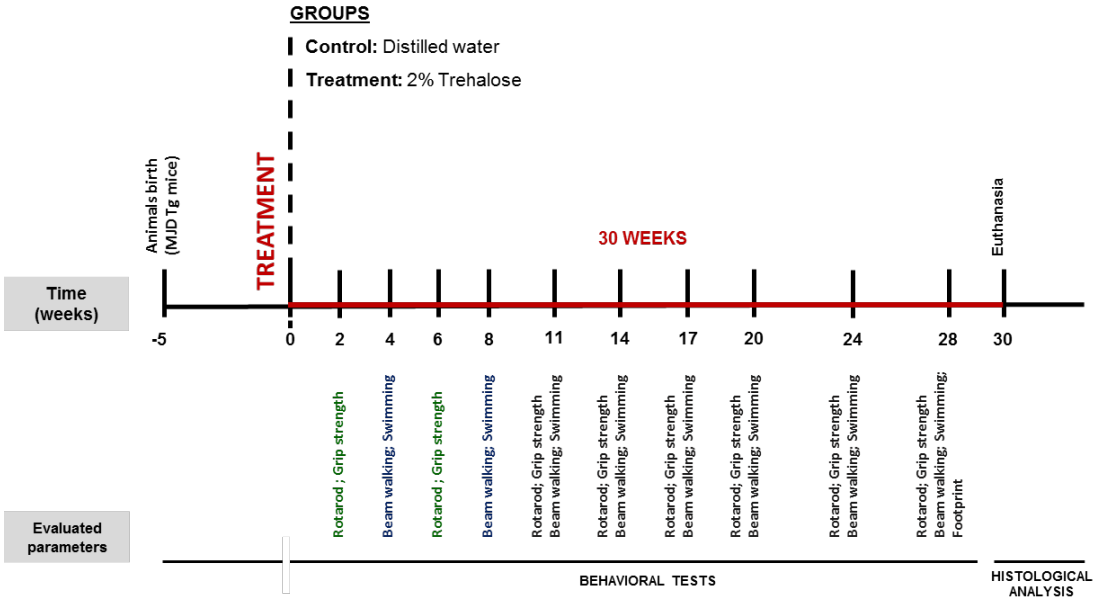


Figure 5 – Study timeline.

MJD transgenic mice were divided into Treatment and Control groups that received either 2% trehalose in drinking water or fresh distilled water, respectively, from the age of 5 weeks up to 35 weeks. Motor and neuromuscular function of the animals was tested by performing different behavioural tests (rotarod, beam walking, swimming and grip strength test) at 5 weeks of age, before the beginning of trehalose treatment (t=0) and afterwards it was evaluated at several time points (t=2, t=4, t=6, t=8, t=11, t=14, t=17, t=20, t=24, t=28 weeks of treatment. The footprint analysis was performed only at t=28 weeks of treatment. The animals were sacrificed at 35 weeks of age (t=30) and brain tissues were collected to perform histopathological analysis of the cerebellum.

2.1.3.1. Rotarod performance test

Motor coordination and balance were evaluated in a rotarod apparatus (Letica Scientific Instruments, Panlab, Barcelona, Spain). Mice were placed on the rotarod at a constant speed

(5rpm) and at accelerated speed (4 to 40 rpm in 5 minutes) and the latency to fall off the rotating rod was recorded for a maximum of 5 minutes. Mice were allowed to perform four trials for each test and time point, with at least 30 minutes rest between trials. For statistical analysis, the mean latency to fall off the rotarod of 4 trials was used.

2.1.3.2. Grip strength test

Grip strength test was performed to assess mice neuromuscular function. The grip strength of forelimbs was determined using a device consisting on a 300g metal grid on a scale. The strength was determined as the animal pulled the grid (g) from the scale with its forepaws. The test was performed during 10-15 consecutive trials and the mean of the four best performances was chosen for analysis. Mice body weight was used as a normalization factor.

2.1.3.3. Swimming

Swimming test allowed the evaluation of motor coordination and strength. Mice were placed on one extremity of a tank filled with water at room temperature and allowed to swim along a 60-cm-long, 14-cm-wide tank until they achieve the platform (located at the other extremity and at the water level). Mice were subjected to 4 trials for each time-point, with a minimum of 30 minutes rest between trials. The mean latency time (to swim across the pool and climb the platform) of the last three trials was recorded.

2.1.3.4. Beam walking test

Fine motor coordination and balance were evaluated in the beam walking test. Mice were trained to cross an elevated beam to reach an enclosed escape platform. A 60W desk lamp was positioned at the start of the beam to create an aversive stimulus (bright light). Two beams (18mm and 9mm square wide) were raised to a height of 21cm and the mice walked 40cm to reach the escape platform. Mice performed four trials for each beam at each time point. The mean latency time (up to 60s) to cross the beam of the four trials was recorded.

2.1.3.5. Footprint analysis

The footprint test was performed only 28 weeks after treatment and it was used to evaluate gait patterns. Mice's hind and forefeet were coated with blue and red nontoxic paints, respectively, and the animals were then allowed to walk along a 100cm-long, 10cm-wide runaway (with 15cm-high walls) over a fresh sheet of greenish paper. The footprint patterns were analysed for four parameters (in centimetres): stride length, frontbase, hindbase and overlap. Stride length was measured as the average distance of forward movement between each stride. Frontbase width and hindbase width were measured as the average distance between left and right front and hind footprints, respectively. These values were determined by measuring the perpendicular distance of a given step to a line connecting its opposite preceding and proceeding steps. Finally, the distance from left or right front footprint/hind footprint overlap was measured to evaluate uniformity of step alternation. When the center of the hind footprint matched exactly with the center of the preceding front footprint, a value of zero was recorded. When the footprints did not overlap, the distance between the center of the footprints was recorded. A sequence of five consecutive steps was chosen for evaluation, excluding footprints at the beginning and at the end of the run. Measurements were all made by the same operator. The mean value of each set of five was considered for each animal.

2.2. Histological processing

2.2.1. Tissue preparation

After 30 weeks of treatment, the animals were given an avertin overdose (2.5×200mg/g, i.p.) and were transcardially perfused with a phosphate solution (0.1M) followed by fixation with 4% paraformaldehyde (PFA, Fluka, Sigma, Buchs, Switzerland). The brains were removed, post-fixed in 4% PFA for 24 hours at 4°C, and then cryoprotected by immersion in 25% sucrose/phosphate buffer for 48 hours at 4°C. The brains were frozen at -80°C and then the entire cerebellum was sliced in 30 μM midsagittal sections using a cryostat-microtome (CM3050S, Leica Microsystems). Sections were collected in anatomical series and stored at 4°C as free-floating sections in PBS supplemented with 0.05M sodium azide until processing.

2.2.2. Neuropathological evaluation

2.2.2.1. Immunohistochemical procedure

The immunohistochemical procedure was performed in a free-floating system, as referred elsewhere (Simões *et al*., 2014). Briefly, sections were simultaneously blocked and permeabilized with 0.3% Triton X-100 in PBS supplemented with 10% normal goat serum (NGS, Gibco) for 1 hour at RT. Sections were incubated overnight at 4°C with the following primary antibodies diluted in 0.3% triton/PBS supplemented with 10% NGS: rabbit polyclonal anti-calbindin D-48K (1:1000 Chemicon, Temecula, CA, USA) and mouse monoclonal anti-HA (1:1000 InvivoGen, San Diego, CA, USA). After the incubation with the primary antibodies, sections were incubated with the appropriate secondary antibodies: goat anti-rabbit or goat anti-mouse conjugated to fluorophores (Alexa Fluor 568 and 488, 1:200 Molecular Probes, Oregon, USA) for 2 hours at RT. Afterwards, sections were washed three times in PBS, counterstained with DAPI to perform the nuclei staining and mounted in gelatinized slides. Once dried, slides were mounted with Mowiol reagent (Sigma). Fluorescence images were acquired with a Zeiss Axiovert 200 imaging microscope.

2.2.2.2. Cresyl staining to determine cerebellar layer thickness and volume

Eight sagittal sections on the extent of the mice left hemicerebellum (with an inter-section distance of 240 μM, which corresponds to eight sections of 30 μM) were mounted in gelatine covered microscope slides and dried at RT. Sections were then stained with cresyl violet for 5 minutes, differentiated in 70% ethanol, dehydrated by passing through 95% ethanol, 100% ethanol and xylene solutions, and mounted with EukittH (Sigma-Aldrich).

2.3. Imaging quantification

2.3.1. Quantification of cerebellum layers thickness

To assess the granular and the molecular layers thickness, quantifications were performed in 4 sections per animal (cresyl violet staining) with an inter-section distance of 240 μM. Mosaic pictures were taken using a PALM Laser microdissection microscope (Carl Zeiss, Germany) with a 20x objective. For each section, layers length was blindly determined

using Fiji software. Layers thickness was assessed by measuring the mean width of each layer at interlobular regions. Purkinje cell layer thickness was determined along with the molecular layer.

2.3.2. Quantification of cerebellum volume

Quantification was assessed using 8 sections, with an inter-section distance of 240 μM (cresyl violet staining) in a blind fashion. Mosaic pictures were taken using a PALM Laser microdissection microscope (Carl Zeiss, Germany) with a 20x objective. This parameter was assessed by measuring the area of the cerebellum in each section. Hemicerebellum final volume was extrapolated using the following formula: volume = (area x section thickness) x number of sections.

2.3.3. Quantification of Purkinje cells and mutant ataxin-3 aggregates

Quantitative analysis of Purkinje cells (calbindin-positive cells) and mutant ataxin-3 aggregates (HA aggregates) was performed over eight sagittal sections on the extent of the mice left hemicerebellum (with an inter-section distance of 240 μM, which corresponds to eight sections of 30 μM) per animal.

To calculate the number of Purkinje cells, mosaic pictures were taken using a PALM Laser microdissection microscope (Carl Zeiss, Germany) with a 20x objective. Images were counted manually using appropriate tools from the Image J software, in a blind fashion.

The total number (no) of Purkinje cells per hemicerebellum was calculated by extrapolation, using the formula:

No of Purkinje cells= (no of cells in section 1 + no of cells in section 2 + … + no of cells in section 8) x 8 sections

The total number (no) of HA aggregates was manually counted by an operator in a blind fashion, by visualizing immunostained slices using a Axioskop 2 plus microscope (Carl Zeiss, Germany) and then extrapolated using the following formula:

No of mutant ataxin-3 aggregates= (no of aggregates in section 1 + no of aggregates in section 2 + … + no of aggregates in section 8) x 8 sections

2.3.4. Quantification of mutant ataxin-3 aggregates size

Quantitative analysis of mutant ataxin-3 aggregates size was performed over four saggital sections, with an inter-section distance of 240 μM. Three representative pictures of lobule IX were taken using a Cell Observer Spinning Disk (Carl Zeiss, Germany) with a 100x objective. Z projections were generated and aggregates size was manually determined by an operator in a blind fashion using Fiji software.

2.4. Statistical Analysis

Raw data analysis was conducted using Prism GraphPad software. Outliers were excluded from analysis using Grubb's test. For weight analysis, statistics was performed using the two-way ANOVA test.

For behavioural results, mean values for each animal were calculated and a trend analysis was performed to compare linear regression slopes using the two-tailed *t* test hypothesis. Data were represented as Mean ± SEM. For histological analysis, statistics was performed using the two-tailed Student's *t* test.

Significance was determined according to the following criteria:

P value	Wording	Summary
< 0.0001	Extremely significant	****
0.0001 to 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
> 0.05	Not significant	ns

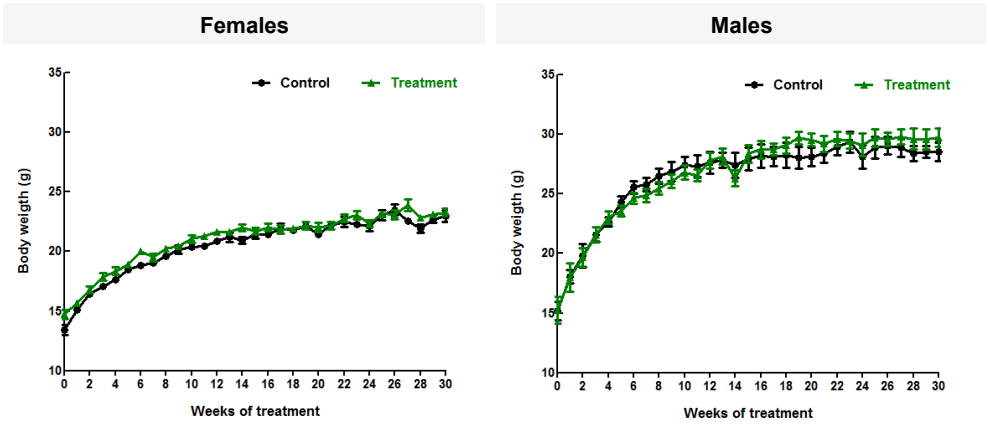
Chapter 3 - Results

3. Results

3.1. No changes were observed in the weight of the animals upon trehalose treatment, although a slight difference was observed in males fur.

Mice body weight was evaluated before the beginning of the treatment (t = 0 weeks) and each week until euthanasia. The physical state of the animals was evaluated daily. No significant differences were observed between treated and control groups (Fig. 6), as observed in other studies involving trehalose treatment (Chen *et al.*, 2015). Regarding general physical health, no relevant changes were observed between groups, except for treated males, who presented a more opaque fur, with an increase in white hair, but with no apparent health impact.

A.



B.

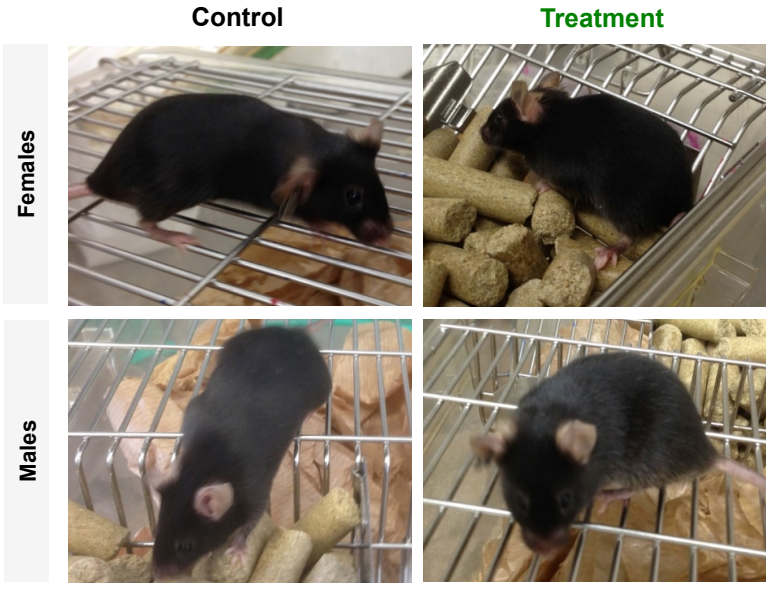


Figure 6 – Body weight and general physical health of MJD transgenic mice treated with 2% Trehalose.

MJD transgenic mice were randomly distributed in two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). Mice body weight and general physical health were evaluated. No statistically significant differences were observed in body weight between control and treatment groups. **(A)**. Trehalose treated-males presented a more opaque fur with an increase in white hair, but with no apparent health impact **(B)**. Statistical analysis was performed using the two-way ANOVA test.

3.2 Trehalose alleviates motor deficits in Machado-Joseph transgenic mice

To evaluate the therapeutic potential of trehalose to recover the behavioural deficits of MJDTg mice, several behavioural tests to assess locomotion and motor function were performed. The behavioural tests started before the beginning of the treatment (t = 0 weeks) and were repeated at different time points over time until 28 weeks of treatment (Fig. 5). At the beginning of the treatment (5 weeks old), the animals already presented a marked phenotype characterized by ataxic movement and difficulty to walk and equilibrate.

To evaluate balance and motor coordination two behavioural tests were performed: the rotarod performance test and the beam walking test. In rotarod performance test, mice were forced to walk in a rotarod apparatus at both stationary (Fig. 7) and accelerated speed (Fig. 8) and the latency time to fall was recorded. A significant improvement in the performance of trehalose-treated females was observed in the stationary rotarod test over time, as showed by an increase in the time that females equilibrated in the rotarod apparatus (Fig. 7B). Beneficial effects started to be noticed after 11 weeks of trehalose administration and were maintained throughout the study. No statistically significant differences were observed in latency time to fall between control and treatment groups, either for males and females, in the accelerated rotarod (Fig. 8 B and C).

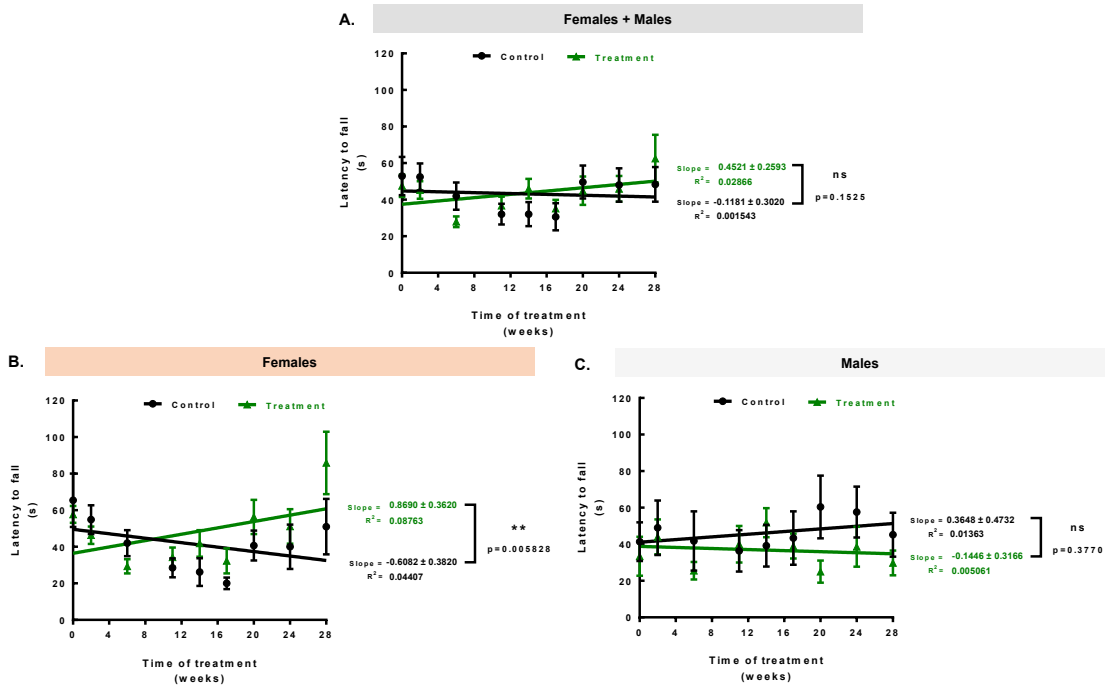


Figure 7 – Stationary rotarod performance test of MJD transgenic mice treated with 2% trehalose.

MJD transgenic mice were randomly distributed into two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). Mice were placed on the rotarod apparatus at a constant speed (5 rpm) for a maximum of 5 min. Latency to fall from apparatus was evaluated before the beginning of the treatment (t=0) and at different time points over time until 28 weeks of treatment (t=28). Trehalose induces a significant improvement in the motor performance of females, as they equilibrate longer in the rotarod apparatus, comparing to control females. Data is presented as Mean±SEM and either combined **(A)** or separately for Females [n=7; **(B)**] and Males [n=5; **(C)**]. Statistical analysis was performed comparing linear regression slopes by a two-tailed *t* test hypothesis: **<0.01; ns= not significant.

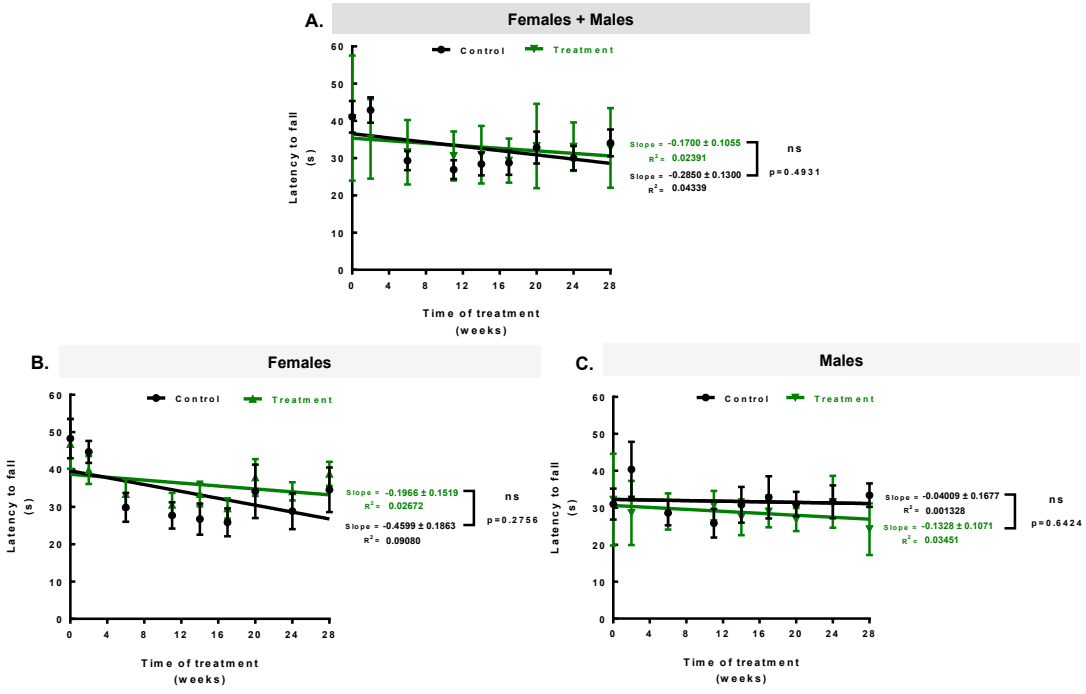


Figure 8 – Accelerated rotarod performance test of MJD transgenic mice treated with 2% trehalose.

MJD transgenic mice were randomly distributed into two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). Mice were placed on the rotarod apparatus at an increased speed (4 to 40 rpm) for a maximum of 5 min. Latency to fall from apparatus was evaluated before the beginning of the treatment (t=0) and at different time points over time until 28 weeks of treatment (t=28). No statistically significant differences were observed in latency time to fall between groups. Data is presented as Mean±SEM and either combined **(A)** or separately for Females [n=7; **(B)**] and Males [n=5; **(C)**]. Statistical analysis was performed comparing linear regression slopes by a two-tailed *t* test hypothesis: ns= not significant.

In the beam-walking test, the ability of mice to remain upright and to walk on an elevated and narrow beam without falling was evaluated. Two beams of increasing difficulty (18mm and 9mm square) were used and the latency time to cross these and reach a safe platform was recorded. Although no statistically significant differences between groups were observed in the 9mm square beam, (Fig. 10) trehalose administration induced a significant improvement in the motor performance of males in the 18mm square beam, as shown by the decrease in the time to cross the beam (Fig. 9 C).

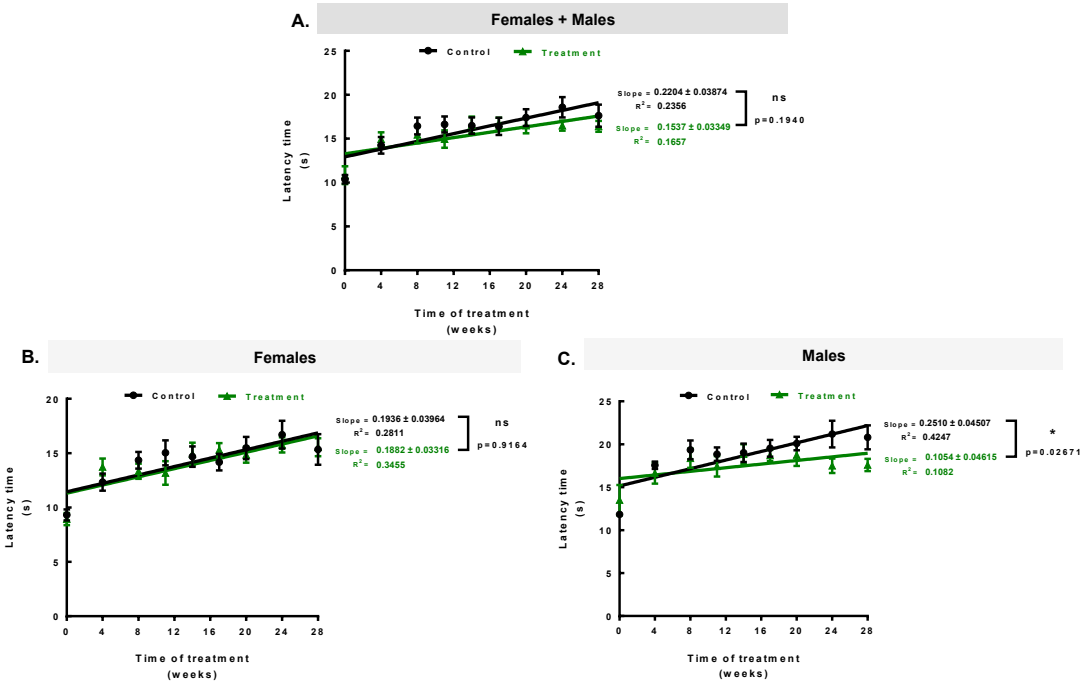


Figure 9 – Beam walking test of MJD transgenic mice treated with 2% trehalose – 18mm square beam.

MJD transgenic mice were randomly distributed into two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). The latency time to cross the 18mm square beam and reach the escape platform was measured. Behavioural assessment started before the beginning of the treatment (t=0) and was repeated at different time points over time until 28 weeks of treatment (t=28).

Trehalose induced a significant improvement in the motor performance of males, as shown by the decreased time of treated animals to cross the beam, compared to controls. Data is presented as Mean±SEM and either combined **(A)** or separately for Females [n=7; **(B)**] and Males [n=5; **(C)**]. Statistical analysis was performed comparing linear regression slopes by a two-tailed *t* test hypothesis: *<0.05.

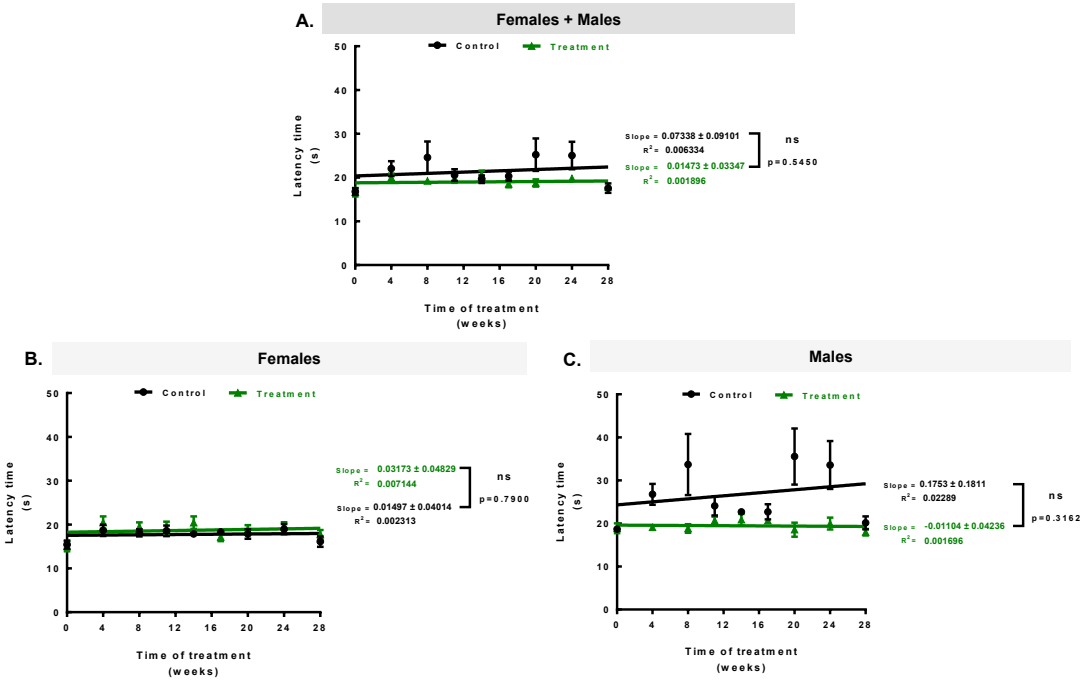


Figure 10 – Beam walking test of MJD transgenic mice treated with 2% trehalose – 9mm square beam.

MJD transgenic mice were randomly distributed into two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). The latency time for mice to cross the 9mm square beam and reach the escape platform was measured. Behavioural assessment started before the beginning of the treatment (t=0) and was repeated at different time points over time until 28 weeks of treatment (t=28). No statistically significant differences were observed between groups. Data is presented as Mean±SEM and either combined **(A)** or separately for Females [n=7; **(B)**] and Males [n=5; **(C)**]. Statistical analysis was performed comparing linear regression slopes by a two-tailed *t* test hypothesis: *<0.05.

Both motor coordination and strength were assessed in the swimming test (Fig. 11). Mice were dropped on the water at one extremity of the swimming pool and the time to swim across the pool and climb the platform was recorded. As shown in Figure 11 C, MJDTg males treated with trehalose took less time to swim across the pool and climb the platform when compared to controls, revealing a significant positive effect of trehalose in the performance of treated males.

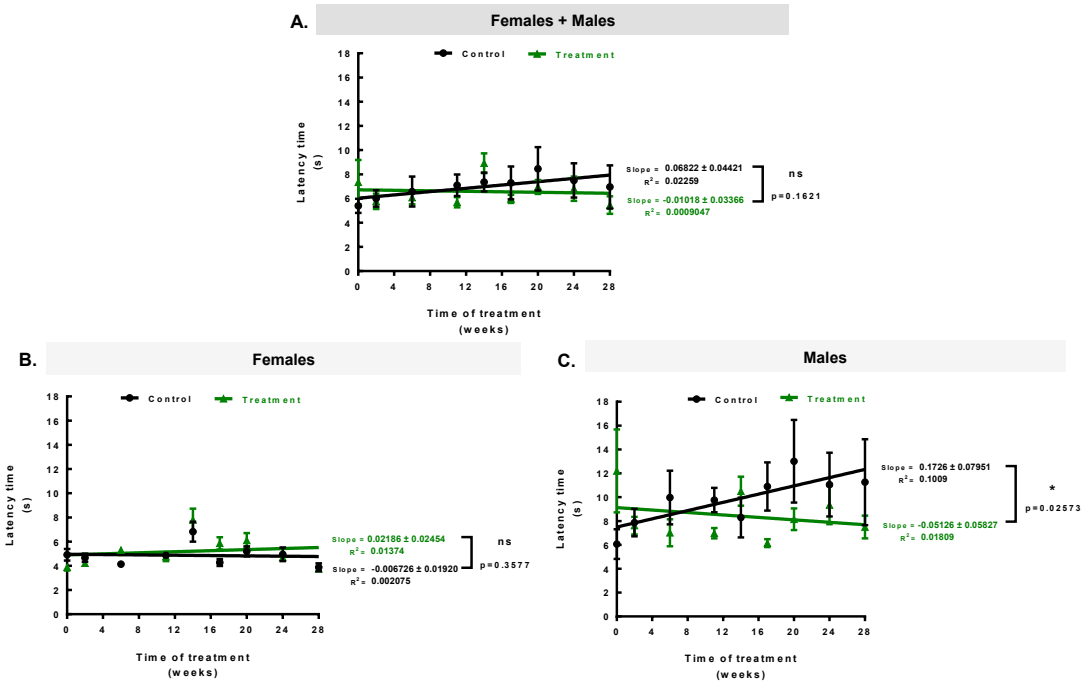


Figure 11 – Swimming test of MJD transgenic mice treated with 2% trehalose.

MJD transgenic mice were randomly distributed into two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). Mice were dropped on the water at one extremity of the swimming pool and the time to swim across the pool and climb the platform was recorded. Behavioural assessment was done before the beginning of the treatment (t=0) and at different time points over time until 28 weeks of treatment (t=28). Trehalose had a significant positive effect in the performance of males, who take less time to swim across the pool and climb the platform when compared to controls. Data is presented as Mean±SEM and either combined **(A)** or separately for Females [n=7; **(B)**] and Males [n=5; **(C)**]. Statistical analysis was performed comparing linear regression slopes by a two-tailed *t* test hypothesis: *<0.05.

A relevant characteristic of Machado-Joseph disease, shared with other SCAs and/or polyglutamine diseases, is the progressive gait and limb ataxia (Carter *et al.*, 1999; Chen *et al.*, 2008; Goti *et al.*, 2004;). MJDTg mice exhibit significant and early onset gait deficits presenting reduced stride length and enlarged front and hindbase (Nascimento-Ferreira *et al.*, 2013). At the end of the treatment, an additional analysis of the footprint patterns was performed to investigate whether trehalose could rescue limb and gait ataxia of MJDTg mice (Fig. 12). As observed in Figure 12C, a significant increase in the stride length and a decrease in the front base (Fig. 12D) parameters were observed in trehalose-treated females compared to controls, showing that trehalose reduced gait deficits of MJDTg females.

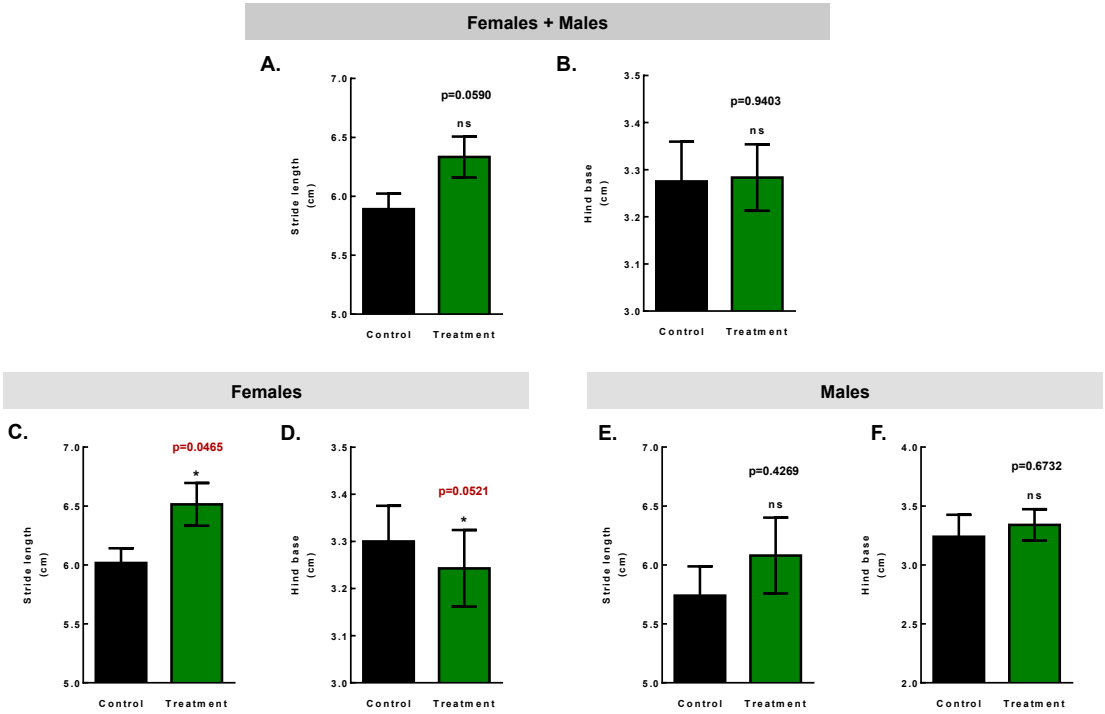


Figure 12 –Footprint analysis of MJD transgenic mice treated with 2% trehalose.
MJD transgenic mice were randomly distributed into two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). Footprint analysis was performed 28 weeks after treatment (t=28). Mice front and forefeet were coated with paint and mice were allowed to walk on a blank greenish paper to obtain footprint patterns. Stride length was measured as the average distance of forward movement between each stride. Front and hindbase was measured between left and right footprints, footprint overlap was measured between the center of left and right front/hind footprints. Trehalose reduced gait deficits of MJD transgenic females, increasing the stride length and decreasing the frontbase distance. Data is presented as Mean±SEM and either combined **(A, B)** or separately for Females [n=7; **(C, D)**] and Males [n=5; **(E, F)**]. Statistical analysis was performed by using the *t* test hypothesis: *<0.05, comparing to controls; ns= not significant.

Overall, behavioural data show that 2% Trehalose treatment reduced motor deficits of both males and females MJDTg mice.

3.3 Trehalose had no effect in recovering neuromuscular function in Machado-Joseph disease transgenic mice

The neuromuscular function is affected in SCA 3 patients, since the primary function of the cerebellum is to maintain the excitability of the motor cortex and the subsequent control of movement (Paulson, 2013; Chen *et al.*, 2015). Therefore, the grip strength test was performed to assess the ability of trehalose to recover neuromuscular deficits in MJDTg mice. As can be shown in Figure 13, no statistically significant differences in forelimb strength

were observed between groups, for both males and females, suggesting that trehalose had no effect in recovering neuromuscular function of MJDTg mice.

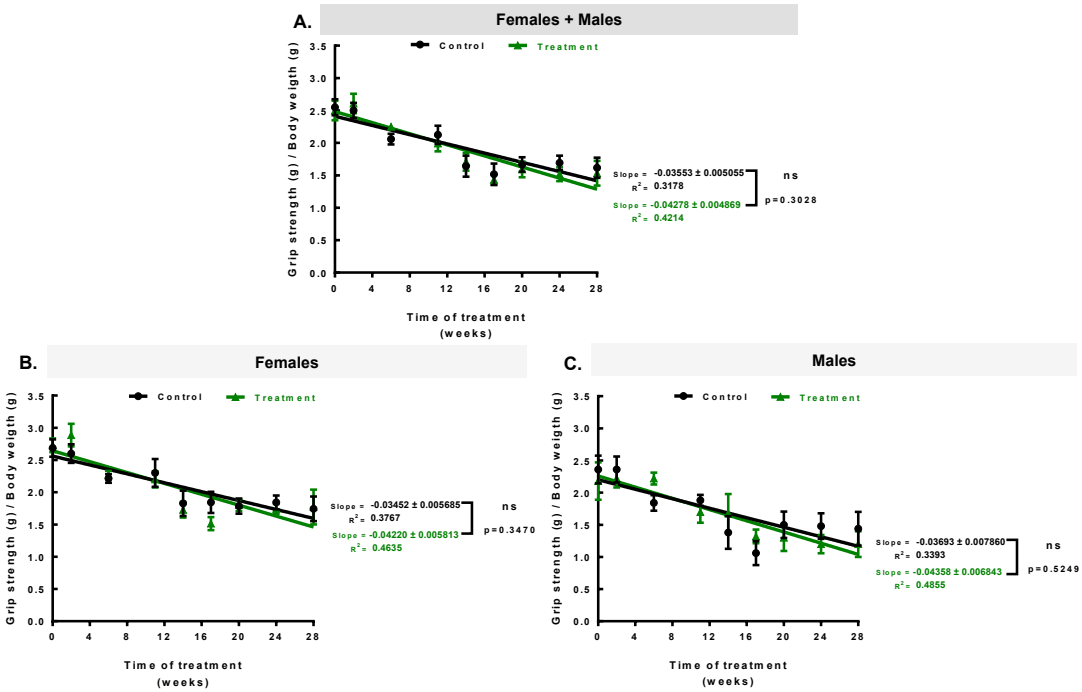


Figure 13 – Grip strength test of MJD transgenic mice treated with 2% trehalose.

MJD transgenic mice were randomly distributed into two groups and then orally treated either with vehicle (Control group; black) or 2% trehalose (Treatment group; green). Mice were placed on the grip strength device and the strength was determined as the animal grasped the net with its forelimbs and the weight was pulled, normalized with the weight of the animals. Grip strength assessment was done before the beginning of the treatment (t=0) and at different time points over time until 28 weeks of treatment (t=28). No statistically significant differences were observed between groups. Data is presented as Mean±SEM and either combined **(A)** or separately for Females [n=7; **(B)**] and Males [n=5; **(C)**]. Statistical analysis was performed comparing linear regression slopes by a two-tailed *t* test hypothesis: ns= not significant.

3.4 Trehalose reduced cerebellar atrophy in Machado-Joseph disease transgenic mice

Cerebellar atrophy, with a reduction of cerebellar layers thickness, is a characteristic of this MJD mouse model, as consequence of the strong cellular connection of the cerebellar cortex, which propagates the degeneration of one cell to the next one (Nóbrega *et al.*, 2013). To determine if the phenotypic improvement was due to a morphologic preservation of the cerebellum, we quantified the total volume of the cerebellum and measured the thickness of both molecular and granular layers of the cerebellum.

Despite no differences between groups were observed for cerebellar volume (Fig. 14), cresyl violet staining revealed a larger thickness and preserved somata organization of cerebellum layers of treated MJDTg mice, when analysed in separate or joint males and females groups,

when compared to controls, suggesting the prevention of neurodegeneration by Trehalose (Fig. 15).

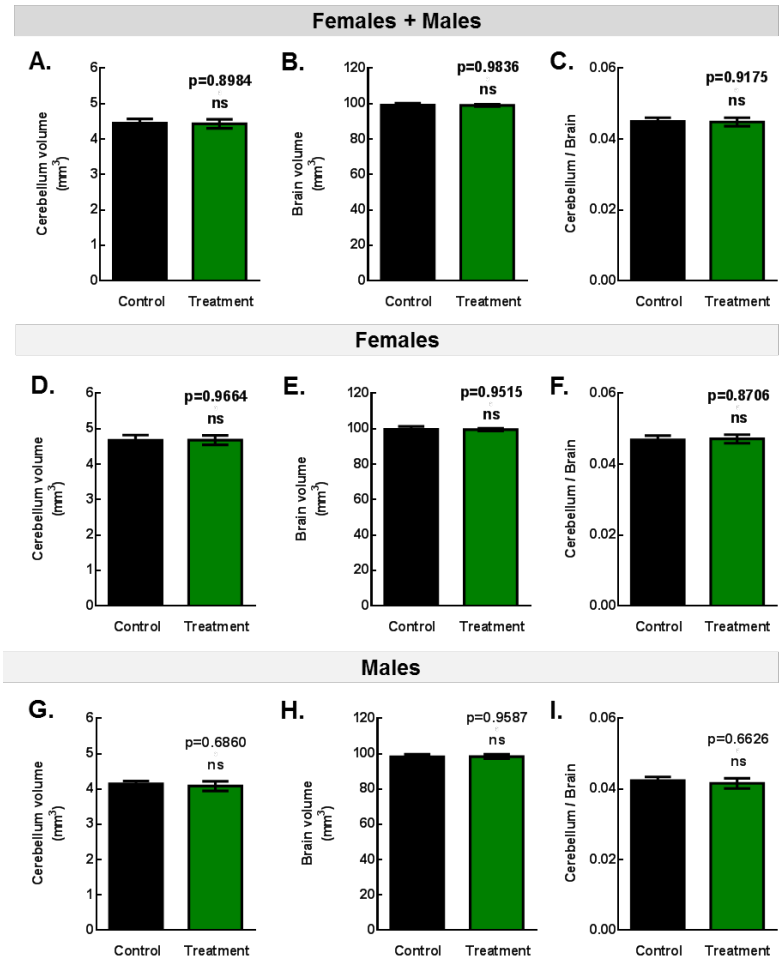


Figure 14 – Cerebellum and brain volume of MJD transgenic mice treated with 2% trehalose.

Cresyl violet staining of left hemicerebellum sections from MJD transgenic mice treated with 2% trehalose (Treatment) and vehicle (Control) for 30 weeks was performed. Brain and cerebellum volume was quantified. No differences were observed in cerebellum size between treated and control groups. Data is presented as mean±SEM and either combined **(A, B, C)** or separately for Females [n=7; **(D, E, F)**] and Males [n=5; **(G, H, I)**]. Statistical analysis was performed using the two-tailed Student's *t* test. ns= not significant.

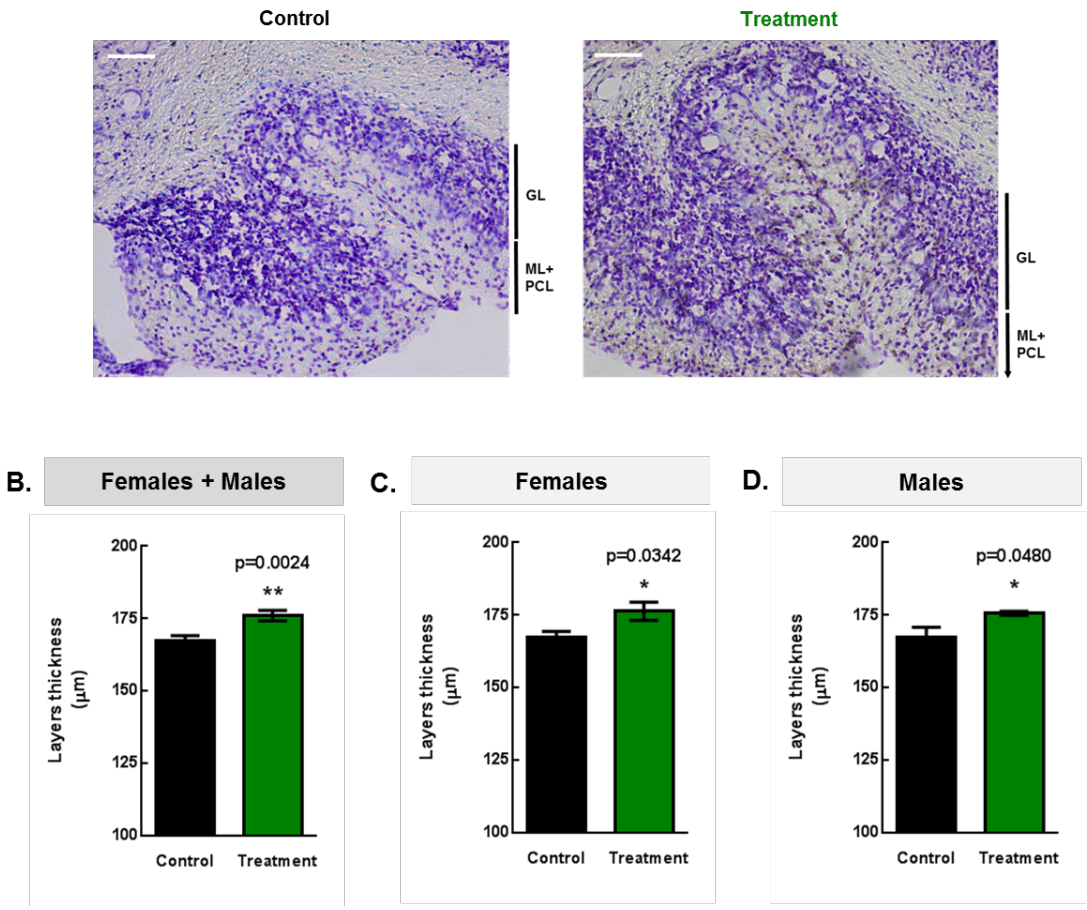


Figure 15 – Cerebellar layers thickness of MJD transgenic mice treated with 2% trehalose. Cresyl violet staining of left hemicerebellum sections from MJD transgenic mice treated with 2% trehalose (Treatment) and vehicle (Control) for 30 weeks was performed. Cerebellum layers thickness was measured **(A)**. Representative picture of cerebellum layers from control and treated MJD females. Trehalose increased the thickness and the somata organization of cerebellum layers, compared to controls. Data is presented as mean±SEM and either combined **(B)** or separately for Females [n=7; **(C)**] and Males [n=5; **(D)**]. Statistical analysis was performed using the two-tailed Student's *t* test. *<0.05, **<0.01, comparing to controls; ns= not significant. Abbreviations: GL, Granular layer; ML, Molecular layer; PCL, Purkinje cell layer. Scale = 50µm.

3.5 Trehalose reduced the size of mutant ataxin-3 aggregates in Purkinje cells of Machado-Joseph disease transgenic mice

Purkinje cells are essential in the cerebellar role of regulating motor function and are significantly affected in MJD patients (Scherzed *et al*., 2012), as well as in this transgenic mouse model, which preferentially express mutant ataxin-3 in Purkinje cells of cerebellar cortex (Torashima *et al*., 2008).

Therefore, we evaluated if the treatment with trehalose would prevent the degeneration of the Purkinje cell layer by quantifying the number of these cells in the cerebellar cortex of MJDTg mice. The immunostainning against calbindin, a rapid endogenous calcium-buffering protein expressed in Purkinje cells and a determinant of normal motor

coordination and sensory integration (Barsky *et al.*, 2003), revealed no differences in the number of these cells between Trehalose-treated and control animals (Fig. 16 B, E and H).

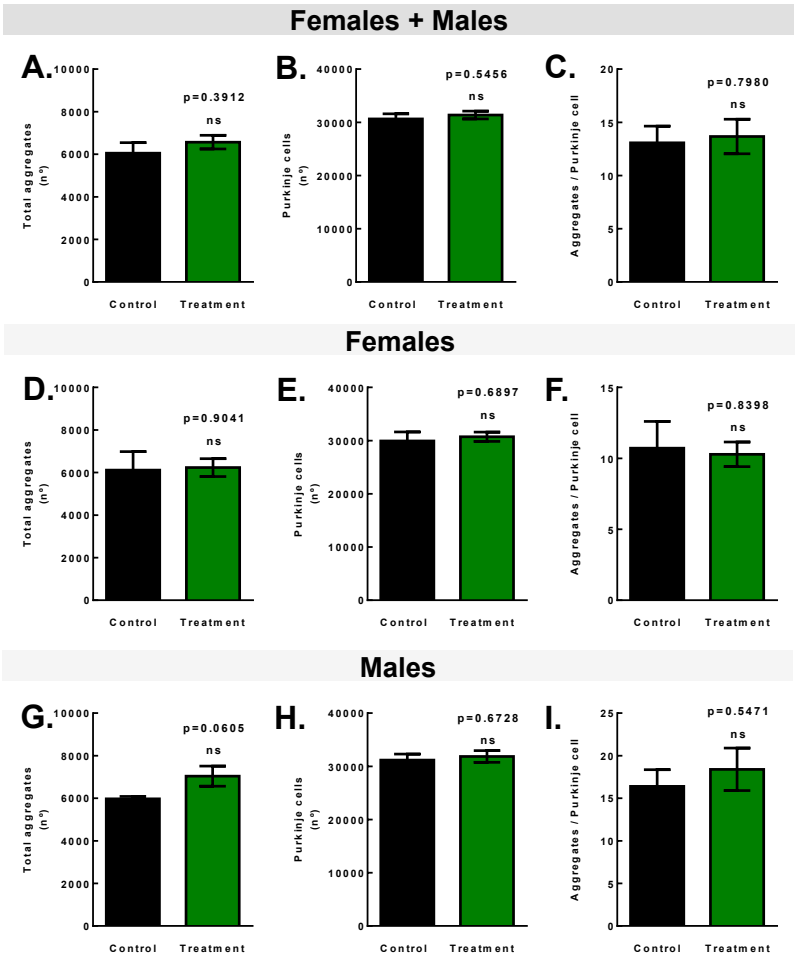


Figure 16 – Number of aggregates and Purkinje cells of MJD transgenic mice treated with 2% trehalose.

Immunofluorescent staining against HA and Calbindin of left hemicerebellum sections from MJD transgenic mice treated with 2% trehalose (Treatment) or vehicle (Control) was performed. Aggregates and Purkinje cells were counted. No significant differences in aggregates and purkinje cell numbers between 2% trehalose-treated and control mice. Data is presented as mean±SEM and either combined **(A, B, C)** or separately for Females [n=7; **(D, E, F)**] and Males [n=5; **(G, H, I)**]. Statistical analysis was performed using the two-tailed Student's *t* test. ns= not significant.

The expanded polyQ tract in mutant ataxin-3 confers toxic properties to this protein and leads to the formation of neuronal intranuclear inclusions that are a hallmark of MJD (Schmidt *et al.*, 1998). We further investigated if trehalose treatment would reduce the number of aggregates in the cerebellum. To evaluate the number and size of mutant ataxin-3 aggregates we performed an immunostaining for HA, since in this model Purkinje cells present aggregates of mutant ataxin-3 tagged by an N-terminal HA epitope (Torashima *et al.*,

2008). We counted the total number of aggregates in the whole cerebellum but no differences were observed between 2%-trehalose-treated and control animals (Fig. 16 A, D and G). Apart from the aggregates number, its size may be implicated in polyglutamine diseases, including MJD. We measured the size of aggregates in lobule IX and we observed that the treatment with trehalose decreased the size of mutant ataxin-3 aggregates (Fig. 17).

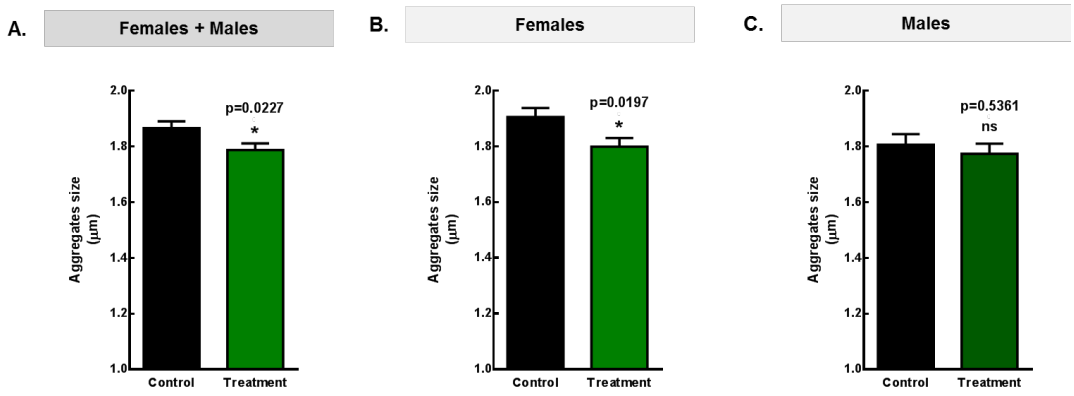


Figure 17 – Size of aggregates of MJD transgenic mice treated with 2% trehalose.

Immunofluorescent staining against HA of left hemicerebellum sections from MJD transgenic mice treated with 2% trehalose (Treatment) or vehicle (Control) was performed. Aggregates size in lobule IX was measured. Trehalose decreased the size of aggregates in lobule IX **(A)**. Data are presented as mean±SEM and either combined **(A)** or separately for Females [n=7; **(B)**] and Males [n=5; **(C)**]. Statistical analysis was performed using the two-tailed Student's *t* test. ns= not significant.

Overall, these results indicate that trehalose has a neuroprotective effect in this transgenic mouse model of MJD.

Chapter 4 – Discussion

Discussion

In this work we assessed whether trehalose alleviated the phenotype of a transgenic mouse model of Machado-Joseph disease. Our data provide evidence that trehalose improved motor impairments and cerebellar neuropathological features in this mouse model for MJD.

Trehalose is a disaccharide that generally exists in yeast, bacteria and invertebrates and has been described as a stabilizer of biomolecules. It is produced among these living organisms in response to stress conditions, allowing their survival (Richards *et al*., 2002). Its stabilization properties are not fully understood, but the beneficial effects of trehalose were described for several diseases, including protein-misfolded disorders. Trehalose alleviated pathological features and behavioural impairments in models of Huntington, Parkinson and Alzheimer's disease, and also in disease models of ALS and prion diseases (Aguib *et al*., 2009; Béranger *et al*., 2008; Castillo *et al*., 2013; Du *et al*., 2013; Liu *et al*., 2005; Sarkar *et al*., 2007; Tanaka *et al*., 2004; 2014; Zhang *et al*., 2014). It has been recently reported that trehalose can alleviate the neuropathological features associated with cerebellar ataxias in SCA 14 and SCA 17 and ameliorate the phenotype of a mouse model of SCA 17 (Chen *et al*., 2015; Seki *et al*., 2010).

As for this experiment, we studied the effects of trehalose in alleviating the behavioural impairments of a MJD transgenic mouse model with expression of a truncated form of human ataxin-3, with 69 glutamine repetitions, driven by a Purkinje-cell-specific L7 promoter (Torashima *et al*., 2008).

The cerebellum, severely atrophied in this model, has an important role in the maintenance of balance and posture, and also in motor coordination. The functional defect of the Purkinje cells in this model implies cerebellar ataxia, including impairments in balance and defects in the gait pattern. The ataxic phenotype in these mice is observed since the 3rd week of age (Torashima *et al*., 2008).

To appraise the therapeutic potential of trehalose we tested motor and neuromuscular function of treated and non-treated mice, performing several behavioural tests. Clear differences were observed between male and female mice. In fact, from our results we speculate that the disease progression is distinct between both genders, as they performed differently in the behavioural tests, and males exhibited a more pronounced phenotype with worse performances than females at each timepoint. Treated female mice stayed for longer

periods in the rotarod apparatus while in constant acceleration, indicating an improvement in balance and motor coordination. Their results in the footprint analysis were also indicative of a rescued gait and limb ataxia, with an improvement in the stride length and in the frontbase width. As for accelerated speed, no differences were observed between treated and control group, probably due to the high degree of difficulty of the test and the skills needed to equilibrate in the rotarod apparatus in this condition. Regarding the results of treated-male mice, there were improvements in the swimming test, indicating a recovery in motor coordination and strength. Their fine motor coordination was also improved by trehalose, as it was demonstrated by the beam walking test. Treated males took less time to cross the 18mm square beam, meaning they recovered their motor skills.

In our study, trehalose has shown clear signs of alleviation of the motor impairments characteristic of MJD. Even though administration of this disaccharide was not able to revert completely the MJD phenotype (particularly the neuromuscular function) probably due to the phenotypic severity of this model since the 3rd week of age, our studies are indicative of a promising option for the treatment of this fatal disease.

Regarding cerebellar neuropathological features, this model presents severe cerebellar atrophy and mutant ataxin-3 intranuclear inclusions, along with Purkinje cell layer disarrangement and degeneration, so it can be useful to correlate the improvement of the neuropathological features with the rescue of the phenotype, while treating the animals with trehalose.

In accordance with previous studies that reported the effect of trehalose in ameliorating the neuropathology of several neurodegenerative diseases (Aguib *et al*., 2009; Béranger *et al*., 2008; Castillo *et al*., 2013; Chen *et al*., 2015; Davies *et al*., 2006; Du *et al*., 2013; Liu *et al*., 2005; Perucho *et al*., 2012; Sarkar *et al*., 2007; Sarkar *et al*., 2014; Seki *et al*., 2010; Tanaka *et al*., 2004; Zhang *et al*., 2014), we also observed an improvement in the MJD associated neuropathology as a result of trehalose treatment.

To associate the rescue of the cerebellar atrophy with possible morphological modifications of specific cellular layers, we measured the thickness of cerebellum molecular and granular layers and analysed the total volume of the cerebellum. Despite no differences between groups were observed for cerebellum volume, cresyl violet staining revealed a larger thickness and preserved somata organization of cerebellum layers of treated MJDTg mice, suggesting a recovery in the cerebellum atrophy characteristic of this mouse model that may be related to the improvements in the phenotype seen in the behavioural tests.

Purkinje cells play a critical role in the regulation of motor coordination and motor learning and, in spite of their extreme importance in cerebellar function they are vulnerable and can be easily damaged by mild insults (Oue *et al*., 2009; Sarna and Hawkes, 2003). This mouse model of MJD presents intranuclear inclusions of mutant ataxin-3 in Purkinje cells since 40 days of age, and their number and size is markedly increased at 80 days of age (Torashima *et al*., 2008).

Neuronal intranuclear inclusions are detected in patients with MJD and other polyQ diseases, and are considered responsible for polyglutamine-mediated neuronal cell death (Paulson *et al*., 1997; Schmidt *et al*., 1998). Nevertheless, the role of aggregates in the pathology of polyA remains controversial. Although a reduction in the number of inclusions by trehalose has been reported in some models of neurodegenerative diseases, such as ALS, Huntington, prions and Parkinson diseases (Aguib *et al*., 2009; Castillo *et al*., 2013; Sarkar *et al*., 2007; Zhang *et al*., 2014), this effect has not been consistently observed. In a mouse model of SCA17, trehalose significantly improved in animals' behavioural performance, but no significant changes were observed in the aggregates number (Chen *et al*., 2015).

In agreement, we observed no differences in the number of aggregates nor Purkinje cells of trehalose-treated animals.

It has been suggested that trehalose may activate protein clearance mechanisms, namely autophagy (Aguib *et al*., 2009; Castillo *et al*., 2013; Sarkar *et al*., 2007; Zhang *et al*., 2014), and this pathway was recently proved to be impaired in MJD patients and in rodent models of this disease (Nascimento-Ferreira *et al*., 2011). Further studies will be required to unravel whether trehalose is activating autophagy in this MJD animal model.

Interestingly, we further analysed the size of mutant ataxin-3 aggregates in lobule IX, and trehalose significantly reduced the size of these species. Regarding studies using a lentiviral mouse model of MJD, Simões *et al*. (2014) recently suggested that despite the detrimental effects of polyQ fragments, larger aggregates presented increased cytotoxicity as compared with smaller ones (Simões *et al*., 2014). Although neuronal intranuclear inclusions are associated with neurodegeneration and cell death among polyQ diseases (Paulson *et al*., 1997; Schmidt *et al*., 1998), others suggest that aggregates formation may be a protective mechanism towards the cytotoxicity mediated by polyQ oligomers (Takahashi *et al*., 2008).

Regarding the transgenic mouse model used in this study (Torashima *et al*., 2008), Oue *et al*. (2009) speculated that the formation of inclusion bodies containing the mutant ataxin-3

could actively recruit polyQ, reducing its transfer to the nucleus of Purkinje cells, where it would be a toxic influence. On the other hand, the formation of inclusion bodies containing mutant ataxin-3 could enhance cytotoxicity as other essential proteins are also recruited to the formation of these species (Oue *et al*., 2009), and in fact Torashima and colleagues (2008) showed that the clearance of polyQ aggregates in Purkinje cells led to the amelioration of its function, with an improvement of the ataxic phenotype of these mice (Torashima *et al*., 2008).

Trehalose reveals molecular chaperone activity, suggested by recent studies that indicate that it has the capacity to assist misfolded proteins back to native-like species (Melo *et al*., 2003), protecting them from denaturation and aggregation and thus maintaining its normal functions under extreme environmental stresses (Borges *et al*., 2002; Santos and da Costa, 2001; Singer and Lindquist, 1998).

Several interaction models have been proposed, and are based in the binding of trehalose to a folding protein, through hydrogen bonds, promoting a stabilization of the folded state, which shifts the reaction equilibrium towards the native state of the protein, leading to a correct protein folding (Allison *et al*., 1999: Colaço and Roser, 1995; Sarkar *et al*., 2014;). In the case of aggregate-prone diseases, the prevention of hydrophobic interactions between mutant proteins leads to an impediment on the formation of aggregates and oligomers (Seki *et al*., 2010), generating a beneficial effect among these diseases.

Tanaka *et al*. (2004) suggested that the inhibition of aggregation by trehalose was due to the interaction with expanded polyglutamines leading to the prevention of further assembly of truncated protein rather than by reversing aggregates formation (Tanaka *et al*., 2004) and the same scenario was suggested for trehalose studies in prion disease by Béranger and col. (Béranger *et al*., 2008). It was also suggested by Tanaka and collaborators (2004) that the increase in the stability of polyglutamine-containing proteins mediated by trehalose could enhance its resistance to proteolysis by caspases and possibly inhibit the translocation of the cleaved fragments to the nucleus.

Since we observed no difference in the aggregates number, but we detected a decrease in the aggregates size, we propose a probable action of trehalose as a chemical chaperone, leading to the stabilization of the polyQ proteins. The promotion of a correct folding by trehalose slows or even hampers further aggregation of mutant ataxin-3 resulting in an important decrease in cytotoxicity. According to our hypothesis, trehalose revealed a protective effect in this transgenic mouse model of MJD.

Mice's weight was assessed every week and trehalose did not cause a significant change between treated and control groups. Regarding general physical health, no relevant changes were observed between groups, except for treated males, who presented a more opaque fur, with an increase in white hair, but with no apparent health impact, yet this effect was not reported until now.

Regarding the water-drinking level, treated mice had a higher consumption in trehalose solution than control mice had of water (data not shown). This scenario was already reported by Chen *et al.* (2015) in the study of the effect of this drug in spinocerebellar ataxia type 17, and we too speculate that it is due to the sweet taste of the trehalose solution.

Oral trehalose is, at least in part, hydrolyzed by trehalase to glucose monomers (Muller *et al.*, 2013). Although trehalase is present in humans and other animals in the intestinal mucosa, in the proximal tubules of the kidneys, in the liver and in blood plasma (Emanuele, 2014), in a study using a transgenic mouse model of Huntington's disease it has been shown that orally administered trehalose reaches the brain tissue, and may be responsible for the observed neuroprotective effects (Tanaka *et al.*, 2004). Nevertheless, in human patients, trehalose is metabolized in the intestinal mucosa in a great extent (Gudmand-Høyer and Skovbjerg, 1996), so in humans the intravenous administration is necessary to reach the brain and mediate the therapeutic effects reported in this thesis. The current use of trehalose in pharmaceutical formulations (including parenteral formulations) proves its safety and the growing interest in this molecule, as it is able to reach intact its target tissues.

In conclusion, trehalose may be considered a promising therapeutic approach to treat MJD, as well as other polyQ disorders.

Conclusion and future perspectives

In this work it was shown that trehalose could alleviate motor impairments and neuropathological hallmarks of MJD, proving that it is a good candidate for the pharmacological treatment of this disease. The current use of trehalose in solid and parenteral formulations as well as in food industry and cosmetics proves its safety and the growing interest in this non-reducing sugar.

Despite our studies have shown the beneficial effects of this disaccharide in a MJD mouse model, the mechanism by which trehalose mediates these improvements remains to be elucidated. As so, in future studies we will investigate thoroughly the mechanisms by which trehalose alleviates MJD neuropathology, using *in vitro* models of MJD. As experimental models we will use a neuroblastoma cell line and also neural stem cells derived from iPS cell lines from MJD patients' fibroblasts.

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