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Ema Ferreira Leal

Machado-Joseph disease: understanding the concepts behind the new therapeutic strategies

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Machado-Joseph disease: understanding the concepts behind the new therapeutic strategies

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Abstract

Machado-Joseph disease, also known as spinocerebellar ataxia type 3, is one of the most common spinocerebellar ataxias worldwide and the second most common polyglutamine disease next to Huntington's disease. Similarly to most polyglutamine diseases, Machado-Joseph disease is an autosomal dominant neurodegenerative disorder caused by the over-repetition of a CAG triplet within the coding region of MJD1 gene. This gene encodes ataxin-3 protein, which is ubiquitously expressed in cells and tissues and, among other functions, thought to participate in cellular protein quality control pathways as deubiquitinating enzyme. The CAG repeat expansion results in an abnormally long polyglutamine tract within the ataxin-3 protein promoting an altered conformation of the protein, leading it to misfold and aggregate as well as conferring it a neurotoxic gain of function through several mechanisms, still not fully understood.

The aim of this review is to bring together updated knowledge on Machado-Joseph disease, to expose some of the cellular pathways so far enlighten through which the mutant ataxin-3 interacts and promotes neurodegeneration and finally to display some of the emerging therapeutic strategies that are being conceived in order to slow down disease progression, to mitigate associated phenotype and even block the expression of the mutant protein.

Keywords: ataxin-3; polyglutamine disease; spinocerebellar ataxia type 3; proteolysis; cleavage fragment; mitochondrial dysfunction; autophagy.

Abbreviations: ALS – amyotrophic lateral sclerosis; AON - antisense oligonucleotides; Atx3Q148 - Ataxin-3 with 148 glutamines; BH3 - Bcl-2-homology-3; CAT – catalase; CDK5 - cyclin-dependent Kinase-5; CHIP – C-terminus of Hsc70 interacting protein; CK2 - casein kinase 2; CNS – central nervous system; DUB(s) – deubiquitinating enzyme(s) ; EPO - external progressive ophthalmoplegia ; ER- endoplasmic reticulum; ERAD - endoplasmic reticulum-associated degradation; GSH-glutathione; GSH-px glutathione peroxidase; GSSG-R - glutathione disulfide reductase; HD - Huntington's disease; HDAC - histone deacetylase; Hsp – heat shock protein; MJD - Machado-Joseph disease ; mtDNA-mitochondrial DNA; mTOR - mammalian target of rapamycin; NES - nuclear export signals; NI - nuclear inclusions; NLS - nuclear localization signals; NPC - nuclear pore complex; NSC- neural stem cells; PD- Parkinson's disease; PLK - Polo-like Kinase-1; PNAs - peptide nucleic acids; PolyQ - polyglutamine; ROS - reactive oxygen species; SB- Sodium butyrate; SBMA - spinal-bulbar muscular atrophy ; SCA3 - spinocerebellar ataxia type 3 ; shRNA- short-hairpin RNAs; Smac - second mitochondria-derived activator of caspase; SNP - single nucleotide polymorphism; SOD -superoxide dismutase ; ST - staurosporine ; Ub – ubiquitin; UIM - ubiquitin-interacting motifs; UPS - ubiquitin proteasome system; VCP - valosin-containing protein; VPA - Valproic acid

Resumo

A doença de Machado-Joseph, também conhecida por ataxia espinocerebelosa do tipo 3, é uma das ataxias espinocerebelosas mais frequente mundialmente e a segunda mais prevalente do grupo das doenças de poliglutamina, a seguir à doença de Huntington. Como a maioria das doenças de poliglutamina, a doença de Machado-Joseph é um distúrbio neurodegenerativo de transmissão autossómica dominante que resulta da expansão de um tripleto CAG de uma das sequências codificantes do gene MJD1. Este gene codifica a proteína ataxina-3, uma proteína ubíqua em células e tecidos que, entre outras funções, pensa-se participar nas vias celulares de controlo de qualidade proteica como uma enzima de ubiquitinação.

A expansão da repetição do tripleto CAG resulta num trato anormalmente longo de poliglutamina dentro da ataxina-3, promovendo uma alteração na conformação da proteína levando-a a um enovelamento incorreto que propícia a sua agregação e lhe confere um ganho de função neurotóxico através de vários mecanismos, ainda não totalmente compreendidos.

O objectivo desta revisão é reunir o conhecimento atual sobre a doença de Machado-Joseph, expor o estado atual do conhecimento sobre algumas das vias celulares através das quais a ataxina-3 mutante atua e promove a degeneração neuronal e por último, revelar algumas das estratégias terapêuticas emergentes que estão a ser concebidas com o objectivo de lentificar a progressão da doença, atenuar o fenótipo associado e mesmo bloquear a expressão da proteína mutante.

Palavras-chave: ataxin-3; polyglutamine disease; spinocerebellar ataxia type 3; proteolysis; cleavage fragment; mitochondrial dysfunction; autophagy.

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Introduction

Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is an autosomal dominant inherited neurodegenerative disorder. Although it was first described amongst individuals of Azorean descent, and later on thought to be geographically related to the Portuguese discoveries, currently it's known to exist worldwide and to be present in many ethnic backgrounds (1-3). It represents the most common form of spinocerebellar ataxias (1) and is comprised in a class of diseases collectively known as polyglutamine (polyQ) diseases, which is a group of fatal neurodegenerative disorders caused by a trinucleotide (CAG) repeat expansion that is translated to an abnormally elongated glutamine tract within the corresponding mutant protein (1,4). There are at least nine known members in this group of diseases and all are inherited in an autosomal dominant manner, except one, the spinal-bulbar muscular atrophy (SBMA), which is X-linked (4). This group of polyQ diseases, in spite of being an heterogeneous group and differing in the regions of the brain affected, they appear to share neurodegenerative pathways and they typically have a late on-set and a progressive clinical evolution profile (1, 4).

MJD presents a strong phenotypic variability, which prompt the organization of patients into three main subtypes, with a common base of cerebellar ataxia and external progressive ophthalmoplegia (EPO), but considerable clinical variability regarding the age of on-set, the neurological features and the degree of disability it entails (1,2,5): Type 1, is characterized by a premature manifestation of the disease (mean age 24,3 years), a more rapid progression of symptoms and presenting marked extrapyramidal features (bradykinesia and dystonia) and pyramidal signs (rigidity and spasticity); type 2, presents a more intermediate expression regarding the age of on-set (mean 40,5 years) and the progression of symptoms. The extrapyramidal and peripheral signs, when present, are mild. Patients with type 2 features may maintain these for long periods of time (5 to 10 years) and eventually preserve this phenotype or evolve, differentiating with time into type 1 or 3, by displaying extrapyramidal or peripheral signs, respectively. Type 3, on the other hand, presents a late on-set (mean 46,8 years) and in addition to the cerebellar ataxia and EPO, it's characterized by important manifestations of peripheral signs, with slight or even none, extrapyramidal and pyramidal signs (1,3,5).

Despite these clinical variabilities, the clinical pattern found constitutes the phenotypic expression of only one mutant gene, the ATXN3/MJD1 gene, located at the chromosome 14q32.1 (1,5). This gene encodes a protein called ataxin-3, mainly a cytosolic protein, ubiquitously expressed and thought, amongst other functions, to be involved in transcriptional regulation and in cellular protein quality control pathways, as a polyubiquitin binding protein with ubiquitin protease activity (1, 6). Despite being ubiquitously expressed, the mutant ataxin-3 causes selective neuronal degeneration in specific brain regions (1, 6). It has been establish in MJD patients an inverse correlation between the size of CAG repeats and the age at onset as well as disease severity - the longer the repeat expansion the earlier the age of onset (2, 4).

The goal of this review is to expose the cellular mechanism considered to be important pathways in the pathology of MJD and the therapeutic strategies currently emerging that have as target this same cellular pathways: proteolytic processing, aggregation, nuclear shuttling, clearance mechanisms and the mitochondrial dysfunction (4).

Proteolytic processing

A common neuropathological feature of polyQ diseases is the accumulation of insoluble intraneuronal protein deposits (inclusions), in specific subset of neurons (7,8). The composition of these inclusions is mainly of the pathological polyQ protein, but also of other components such as molecular chaperones, transcriptions factors, ubiquitin, non-pathogenic polyQ proteins and components of proteasome machinery (7-9). It has been proposed that proteolytic cleavage, as the source of breakdown products, is the initial step in the molecular disease development. This mechanistic concept is known as *the toxic fragment hypothesis*, which predicts that proteolytic production of polyQ–containing fragments from the full-length polyQ expanded disease protein initiates the aggregation process associated with inclusion formation and cellular dysfunction (4,7,8,10).

Taking in consideration the role of proteolytic processing, and that the cleavage of disease proteins by proteases is presumably an important step in the pathogenesis of neurodegenerative diseases, multiple publications are focusing on displaying how modulating the activity of cleavage-responsible proteases or decreasing the levels of toxic fragments could represent a promising therapeutic approach (4,7). There are several classes of endogenous proteases currently linked to the proteolysis of polyQ proteins, including the groups of caspases and calpains (4, 8,11).

Caspases are cysteine proteases mainly associated with apoptotic pathways and inflammation (4,12). These proteases cleave proteins at specific aspartate residues and cleavage is influenced by protein-protein interactions and cell type (11). It has been detected an increase activation of caspases in the course of polyQ diseases, as first evidenced by a report in Huntington's disease (HD) (4,13). Ataxin-3 protein has also been shown to be a target for caspase-mediated cleavage in cell culture and *in vivo* (11,14). Endogenous ataxin-3, as a polyubiquitin binding protein with ubiquitin protease activity, presumably engages in a diversity of protein-protein interactions that may modulate its susceptibility to caspases (4,11). In some reports, which studied the proteolysis of normal and pathogenic ataxin-3 and the role of caspase-mediated cleavage, cells that were undergoing an induced apoptosis, generally treated with staurosporine (ST), led to the appearance of proteolytic fragments of different size, depending on the length of the polyQ repeat (11,14). The cleavage in these situations was increasingly suppressed by zVAD-fmk, a broad-spectrum caspase-inhibitor, and fragment production was reduced as well as the increased aggregation seen in cells with expanded ataxin-3 constructs and treated with ST (11,14). Additionally, reports suggest that caspase-1 is a primary mediator of cleavage, given that in an effort to determine which caspase-mediated ataxin-3 cleavage, caspase-1 inhibitor was the most effective at inhibiting ataxin-3 proteolysis and fragment production (4,11). Furthermore, results in a *Drosophila* model expressing a sextuplet caspase recognition site mutation in Ataxin-3 revealed dramatically reduced amounts of cleavage products, strongly suppressing caspase-cleavage of the protein *in vivo* and strikingly mitigating neural degeneration, despite the fact that nuclear inclusion formation remained unaffected (4,14).

Moreover, a recent publication presented cyclin-dependent Kinase-5 (CDK5) as a potent neuroprotector, shielding Ataxin-3 from caspase cleavage and attenuating caspase-mediated aggregate formation as well as reducing neuronal cell death (15).

The second group of proteolytic enzymes implicated with cleavage of polyQ-expanded proteins is called calpains. These enzymes are a class of calcium-dependent cytoplasmic cysteine proteases, ubiquitously expressed and involved in a multitude of regulatory cellular functions, specializing in modulating structure, localization and activity of their substrates (4, 10). The role of Calpain in the pathogenesis of SCA3 has been investigated in a variety of studies. Even though, initial reports stated that an involvement of calpains in SCA3 was not detectable (11,14) and cleavage by caspases and calpain is still controversially discussed (16), calpain-mediated proteolysis triggered by calcium has been reported both *in vitro* and *in vivo* in ataxin-3, as in HD (8,16). It has been shown that inhibition of calpains reduces cleavage of the mutant ataxin-3, mediates its translocation to the nucleus, the aggregation in intranuclear inclusions, neuronal dysfunction and neurodegeneration (10). The Calpain system is centrally comprised in three distinct important proteins in neurons (16). The two best characterized members of this family are calpain-1 and calpain-2, which are activated by a distinct range of calcium concentration. As the activation of calpains is an irreversible process, it must be strictly controlled (16). Calpastatin is a known endogenous inhibitor of these calpains that binds to the inhibitory domain on both sides of the active site cleft of ataxin-3 in a reversible manner (16). This inhibitor was used in a number of studies to demonstrate how ataxin-3 is a sensitive calpain substrate and how its mediated inhibition of calpains reduces cleavage, nuclear translocation and aggregates formation, preventing cell injury and providing neuroprotection (8,10,16). It has been also determined that ataxin-3 is cleaved more rapidly and efficiently by calpain-2 than by calpain-1 (16). The sequence within ataxin-3 recognized by calpain-2 was identified by microsequencing to be around amino acid residue 260 in Ataxin-3, which seems to be of particular interest since cleavage of polyQ expanded Ataxin-3 in this region is known to produce highly aggregation prone fragments, that can initiate the aggregation process and the recruitment of full-length ataxin-3 into co-aggregates (8). The role of calcium seems to be of particular importance in this calpain-mediated cleavage and evidence of dysregulation of cellular calcium homeostasis in polyQ diseases have been described (8,17,18). Even more, there seems to be an association between expanded ataxin-3 oligomers and glutamatergic receptors, specially AMPA-R (17,18), with data suggesting that depolarization-induced Ca2+ influx, via voltage-gated Ca2+ channels, and the subsequence neuron-specific cascade of events were implicated in protease activation and ataxin-3 cleavage, assumebly being essential for phenotypic manifestations (18).

Agreggation and Fibrillogenesis

Information about the relationship between aggregate-neurotoxicity is quite complex and currently only partially understood (17). The aggregation of ataxin-3 was shown to undergo a two-stage

pathway, where the first stage of the aggregation is independent of the poly-Q domain and produces soluble aggregates, whereas the second stage is a poly-Q length dependent process and is accompanied by a dramatic loss of solubility of the aggregates, an alteration in their stability, producing highly stable aggregates, and morphology of the end-stage fibrils (4, 19).
Ataxin-3 is a 42-kDA protein containing a structured globular N-terminal Josephin domain, with deubiquitinating activity, followed by two or three ubiquitin-interacting motifs (UIM), depending on the protein isoform, and an unstructured and flexible C-terminal region containing a polyQ tract, whose expanded pathological variants are responsible for the disease (7, 17). The repeat threshold of the polyQ stretch is generally accepted to be above ~ 52 repeats to result in disease, leading to a toxic gain of function of the protein, misfolding and aggregation of ataxin-3 (6, 15). Ataxin-3 is ubiquitously expressed in cells and tissues and displays a subcellular distribution involving both the nucleus and the cytoplasm (20, 21), depending on the cell type, which in unaffected brain and normal neurons, appears to be predominantly cytoplasmic (2). It has been suggested that non-expanded ataxin-3 has an intrinsic propensity to aggregate and that the region responsible for this inherent ability to form aggregates involves the N-terminal Josephin domain (19). This propensity of non-expanded ataxin-3 seems to only extend to the first-stage of the aggregation pathway, whereas the second stage of this pathway is an additional step introduced and reliant on the presence of polyQ pathological length (19). As reports suggest that the N-terminal sequence prevents aggregation of the polyQ-expanded protein into SDS-insoluble aggregates (7), which seem to be formed through the summation of smaller aggregates (19), it does not seem to protect non-expanded ataxin-3 from sequestration into these aggregates (7). The presence of normal non-expanded ataxin-3 in nuclear inclusions (NI) in other polyQ and neurodegenerative diseases raised the question if it contributes to the pathology or reflects cellular attempts at repair or protection (6). The activities associated with the N-terminal domain of ataxin-3 were suggested to be of great importance in suppressing the degeneration induced by pathogenic proteins by reducing accumulation and delaying NI formation (6). The role of the UIM motifs and the ubiquitin protease domain, which are integrated in the N-terminal region, were found to contribute to ataxin-3 suppression of polyQ toxicity and results indicated that the ubiquitin-related activities of ataxin-3, specially the ubiquitin protease activity, mitigates neurodegeneration, including the toxicity of the pathogenic ataxin-3 itself (6). However these activities and results seem to be dependent upon proteasome activity, which is required to be normal for the protective activity mediated by ataxin-3 to be present (6).

Nucleocytoplasmic shuttling

As previously mentioned, one of the histopathological hallmarks of SCA3 and other polyQ diseases is the formation of neuronal inclusions, essentially in the nucleus, which appears to be an important site of pathology in SCA3 and other polyQ diseases (20, 22). Nuclear localization of Ataxin-3 was found to be required for the manifestation of symptoms in SCA3 (22,23), where it may contribute to its

pathogenesis by impacting gene expression or by disrupting nuclear organization and function (21). Ataxin-3 is a protein with the ability to shuttle in and out of the nucleus, a competence that is being characterized in several studies and which specificities remain elusive. Depending on their size, molecules can travel through the nuclear pore complex (NPC) by one of two mechanism: small molecules can diffuse passively across the nuclear envelope through open aqueous channels in the pore, whereas the translocation of macromolecules larger than 40-60 kDa is an active process, energy-dependent, which relies on the presence of specific sequence motifs that are identified and selectively transported in a specific direction by carrier proteins of Karyopherin-β family, mainly (12).These specific motifs are called nuclear localization signals (NLS) and nuclear export signals (NES), which in Ataxin-3 were mapped in the C-terminal region and the N-terminal region, respectively (12, 21, 24). As nuclear-associated mechanisms are being implicated in the neuropathogenesis of polyQ diseases, it has been suggested that defects on the nucleocytoplasmic shuttling activity of expanded proteins are implicated in disease development and that modulating global nuclear transport, preventing the accumulation in this subcellular compartment, might be a possible therapeutic strategy to help delay or even reverse neurodegeneration (24). It has been demonstrated in transgenic mouse models that adding an exogenous NLS to an expanded Ataxin-3 with 148 glutamines (Atx3Q148) accelerated the formation of polyQ aggregates and intensified the severity of the phenotype, inducing a premature death. On the other hand, when an exogenous NES was added to Atx3Q148, the expanded protein was driven out of the nucleus and the manifestation of a phenotype was remarkably reduced and delayed (22). Accordingly, in a study made in yeast - where the nuclear import apparatus is highly conserved - and further confirmed in mammalian cells, the absence of a functional NLS disrupted the import ability of ataxin-3 (21). Data also indicates that nuclear export of ataxin-3 is partially mediated by a nuclear export receptor, CRM1, and additionally, that an independent CRM1 nuclear export pathway of ataxin-3 seems to be mediated by the N-terminal region of ataxin-3 (Josephin domain+UIMs) (21). This independent pathway requires the context of the Josephin domain and the UIMs, appearing to be critically dependent on the three-dimensional motif, whose integrity becomes compromised when Josephin domain is physically separated from the UIMs (21).

Other studies show that nuclear localization can be modulated by other mechanisms (20, 23). In one of the publications, a regulatory role of nuclear abundance was attributed to casein kinase 2 (CK2), a nuclear protein kinase that is active in the nucleus, and engages in many cellular processes including replication, transcription, translation and signal transduction (23). Phosphorylation of ataxin-3 mediated by CK-2 at serine residues S340/S352 and S236, within the third and first UIM, respectively, was shown to increase nuclear levels of ataxin-3, nuclear inclusions formation and to stabilize ataxin-3 promoting a slower turn-over of the phosphorylated protein (23). However, the impact on nuclear inclusion formation was shown to be contingent since inhibition of CK2 phosphorylation of ataxin-3, by two selective CK2 inhibitors (DMAT and TBB), did not abolish the occurrence of inclusions (23). This led to the hypothesis that CK2 effect was due to the impact on the half-life of phosphorylated ataxin-3 and its enhanced nuclear presence (23). It was also demonstrated that CK-2 phosphorylation

strengthen the transcriptional activities of ataxin-3, possibly contributing to an increase in the aberrant transcriptional effects of expanded ataxin-3 (23).

Intranuclear localization of ataxin-3 has also been demonstrated to be regulated by specific cellular conditions, in particular, two proteotoxic stressors: heat-shock and oxidative stress, the latter being considered a stress that intensifies during aging and has been linked to many neurodegenerative diseases, including polyQ diseases (20). Such proteotoxic stressors were shown to induce nuclear localization of ataxin-3, leading the protein to accumulate in the nucleus. It was also found that in face of these specific proteotoxic stressors there is an increase of damaged and misfolded proteins, which activates a set of response pathways, such as the heat shock response, in an effort to reduce the number of aberrant proteins (20). Although it remains elusive, results suggested that the regular and basal mechanism described earlier modulating the nuclear localization of ataxin-3, in response to heat-shock were not required and did not regulate the heat-shock induced nuclear localization (20). Accordingly, mutating the UIMs, the ataxin-3 DUB active site and the predicted site of NLS did not alter ataxin-3 nuclear localization following heat-shock conditions (20). The same results were observed when CK2 phosphorylation was inhibited with DMAT (20), contradicting an observation made by another report, which suggested that thermal stress increased nuclear presence of ataxin-3 in a CK2-dependent manner (23). It is currently uncertain whether CK-2 phosphorylation contributes to nuclear translocation of ataxin-3 under these stress conditions (25).

In a study conducted with HEK293T cells under heat-shock conditions, phosphorylation of serine-111 in the JD was observed to be required, though not sufficient by itself, for nuclear localization of ataxin-3, even though, under basal conditions (37^{o}C) it seemed to have no effect on its localization (20). Serine-111 was targeted for resembling the "preferred" sequence of Polo-like Kinase-1 (PLK) phosphrylation site. This enzyme is involved in the phosphorylation and subsequent nuclear translocation of Hsf1, which is a key regulator of the heat shock response that seems to be partially involved in the heat-shock-induced nuclear localization of ataxin-3 (20). However, Plk-1 is not present in the brain, only Plk2 and Plk3 were identified in neurons and represent more optimal candidates for phosphorylation of ataxin-3.

In addition, data also indicated that under different proteotoxic stresses the interactions of ataxin-3 with valosin-containing protein (VCP) and hHR23B, which are two protein quality control effectors, were modulated differentially (20).

In summary, the intracellular localization of ataxin-3 is highly regulated, relying on interactions between internal localization signals combined with protein-protein interactions, specific cellular conditions and posttranslational modifications, still poorly understood (25). Having a clearer understanding of these events can help elucidate the disease's pathogenesis and determine possible targets to therapeutic intervention (4, 25).

Clearance mechanisms- Protein quality control systems

In cells there are distinct protein quality control systems to clear misfolded or faulty proteins and preserve cellular homeostasis. In the refolding and clearance of pathogenic ataxin-3 have been implicated molecular chaperones, the ubiquitin proteasome system (UPS) and autophagy (25). Along with degradation by the UPS, autophagy constitutes the major pathway of organelle and protein turnover (26, 27).

Data has shown that autophagy plays an important role in CNS clearance of misfolded proteins, particularly in diseases characterized by the accumulation of insoluble and aggregation-prone proteins, as it happens in polyQ diseases, Alzheimer's disease and Parkinson's disease (PD)(26). Furthermore, reports have provided evidence that autophagy induction reduces the levels of pathogenic ataxin-3 and its toxicity, altering the progression of the disease (26-28). It has been suggested that autophagy is functional in early stages of disease and that impairments in this pathway are apparent at late stages of disease. In view of this premise, it has been proposed that ubiquitinated ataxin-3 inclusions are targeted to the autophagic machinery by p62, a cytoplasmic autophagic receptor that functions as a cargo-recognizing molecule, and that possibly due to saturation in the clearance capacity of the autophagic machinery to degrade mutant proteins, impairments in this pathway are apparent in late stages of disease (27). Autophagy can be upregulated by inhibiting the mammalian target of rapamycin (mTOR) and by mTOR independent pathways (28).

Neuronal activation of autophagy can be induced by rapamycin, an inhibitior of mTOR, or be directly activated by beclin-1, a haploinsufficient tumor suppressor gene in mice (sharing 98%identity with human beclin-1) and whose activity is inhibited by bcl-2 homologs binding to its BH3 domain (26, 29). Temsirolimus, is a rapamycin ester that is able to up-regulate autophagy by inhibiting the kinase mTOR pathway (28). Ataxin-3 transgenic mice treated with temsirolimus presented reduced levels of mutant ataxin-3, which seemed to be associated with the observed improvement in motor coordination performance. It was also observed a significantly reduced aggregate number in transgenic mice treated with temsirolimus in comparison with placebo treated mice (28). It has been hypothesis that transcriptional dysregulation plays a role in SCA3 pathogenesis and in fact, it has been demonstrated in ataxin-3 transgenic mice that there is a mild disruption in transcription, which was partially relieved by treatment with temsirolimus (28).

It has been shown that beclin-1, a key protein in autophagy clearance pathway and that seems to be essential for cell survival, is decreased in a number of neurodegenerative diseases (26, 27). In fact, it was found that alongside the accumulation of proteins related with the autophagy pathway and autophagossomes in brains of patients with MJD, beclin-1 levels were reduced in fibroblasts of MJD patients and rodent models (27). In a lentiviral-based MJD rat, beclin-1 was found trapped in nuclear ataxin-3 inclusions and it was suggested that this entrapment throughout the course of the disease, might be the cause for the reduced levels of beclin-1 found in the previously mention tissues and

models (27). Studies have shown that overexpression of beclin-1 activates autophagy, stimulating autophagosomal flux, promoting the autophagic clearance of misfolded proteins and that this increase seems selectively more efficient for the mutant ataxin-3 when compared to the wild-type ataxin-3 (26,27). Furthermore, overexpression of beclin-1 has been shown to promote neuroprotection by shielding neurons from accumulation of misfolded proteins (26) improving clearance of aggregated, oligomeric and soluble mutant proteins, ultimately reducing neuronal dysfunction (27). Indeed, beclin-1 overexpression in a lentiviral rat model and severely affected transgenic mice was shown to be able to prevent and partially rescue from the motor deterioration characteristic of MJD (26). In this particular study, beclin-1 effects on motor behavior were found to be dependent on the timing of intervention and disease status. It was found that an early beclin-1 overexpression, before the onset of the disease, was able to rescue from the development of balance skills and motor coordination impairment that feature MJD ataxic phenotype, hampering the development of neuropathology. This rescue was evaluated by a robust clearance of mutant ataxin-3 aggregates, the preservation of Purkinje cell dendritic arborizations and of the width of the molecular layer of the cerebellar cortex (26). In contrast, beclin-1 overexpression in transgenic mice mimicking a late stage of disease led to a partial rescue of the ataxic phenotype, regarding balance, gait and motor coordination and was able to ameliorate the neuropathology, slowing down the progression, though it could not completely prevent the cell damage (26). Thus, up-regulation of beclin-1, genetically or pharmacologically, has been found to represent a promising therapeutic approach for polyQ diseases and other neurodegenerative diseases (26).

As mentioned previously, UPS is one of the major pathways of selective protein degradation. This system targets cytosolic and nuclear proteins for proteolysis using ubiquitin (Ub) as a marker. Ubiquitination of proteins is a multistep process that involves firstly, the activation of Ub by its attachment to ubiquitin-activating enzyme, E1. Secondly, the transfer of Ub to a second enzyme called ubiquitin-conjugating enzyme, E2, that in turn mediates ubiquitin transfer to a third enzyme called ubiquitin ligase or E3 (12). There are several E3 enzymes in the cell that are responsible for the selective recognition of different substrate proteins (12). These E3-Ub ligases mediate the covalent attachment of Ub into lysine residues within target proteins (30). Several E3s regulate their own stability and ability by ubiquitinating themselves, generally targeting themselves for proteasome degradation. However, this destruction can be delayed or even prevented by the action of one or more deubiquitinating enzymes (DUBs) removal of the Ub conjugates (30). Ataxin-3, as a DUB and participant in protein quality control pathways, interacts with several E3 ligases and proteasome shuttle proteins. Some of these E3/shuttle components have been reported to be involved in ataxin-3 polyubiquitination and shuttling to the proteasome degradation, such as E4B/VCP, CHIP/Hsp70 AND E6-AP/Hsp70 (25).

The C-terminus of Hsc70 interacting protein (CHIP) is an Hsp70 cochaperone as well as a known ataxin-3 interacting E3 ubiquitin ligase that has been implicated in several neurodegenerative diseases (4, 25). CHIP and ataxin-3 appear to interact and regulate the activity of each other. Evidence

seems to support the notion that deubiquitination of CHIP by ataxin-3 is coupled to its E3 ligase activity and not only contributes to regulate it within the protein quality control pathway, inactivating CHIP via deubiquitination, but also acts as an editor, editing the Ub conjugates that it forms, ensuring that they're the appropriate length to be efficiently targeted to proteasome degradation (30). In the brains of MJD transgenic mice models, levels of CHIP were found significantly reduced and it seemed that the expansion of the polyQ tract increased the binding between CHIP and mutant ataxin-3 and led to a reduction in CHIP levels (30). Although it remains unclear, it was raised the possibility that this enhanced affinity between CHIP and mutant ataxin-3 altered their functional relationship and inadvertilly caused CHIP to be directed to degradation (30). Alternatively, by interacting with the polyQ expanded fragments present in inclusions and directing them to degradation, CHIP was dragged along, ultimately being cleared alongside the aggregates (30). In another report, it was observed that suppression of CHIP, by genetic reduction or elimination, markedly increasing the level of ataxin-3 microaggregates and accelerated the phenotype of the disease in transgenic mice models of MJD (31). Futhermore, it was suggested that CHIP regulates the level of expanded ataxin-3 in neuronal cells and its solubility in the brain (31). The results have been equivocal and it's still unclear why CHIP is degraded and it's levels found reduced in MJD models.
Another E3-Ub ligase whose activity was demonstrated to be regulated by ataxin-3 is called parkin (30). This E3-Ub ligase is enconded by Parkin gene, the mutated gene most commonly known to result in familial Parkinson's disease (32). As observed with CHIP, levels of parkin were found significantly reduced in transgenic mice models of MJD (30). It was demonstrated that non-pathogenic ataxin-3 interferes with the ability of parkin to ubiquitinate itself and, based on *in vitro* observations, it was proposed that ataxin-3 interacts directly with parkin, hindering the latter activity for self-ubiquitination by binding itself to parkin and blocking the E2 from transferring the Ub onto parkin (30). Instead, ataxin-3 promotes E2 to transfer the Ub onto itself and away from parkin (30). On the other hand, it appears that ataxin-3 only regulates parkin activity, having no effect on the levels of parkin, since the presence or absence of wild-type ataxin-3 was indifferent to the overall parkin levels (30). Yet in the presence of a mutant ataxin-3, the scenario seems to be distinct, with the expanded ataxin-3 promoting the clearance of parkin possibly through the autophagy pathway (30). However, a recent report suggested that parkin appears to be capable of preserving proteasome activity and protect it from inhibition by targeting and promoting the ubiquitination and degradation of misfolded polyQ proteins in multiple cellular compartments (32). Parkin seems to be capable of recognizing misfolded polyQ proteins by its interaction with Hsp70, a Hsp molecular chaperone that is important in the recognition and unfolding of misfolding proteins and that appears to mediate the degradation of such proteins (32). It has been suggested that Parkin may play an important role in stress response pathways by promoting the degradation of misfolded proteins, preventing their accumulation and thus preserving proteasome function (32).
It has been demonstrated that proteasome dysfunction can arise from the accumulation of misfolded cytosolic proteins such as expanded polyQ proteins and that this impairment can lead to failure of

endoplasmic reticulum-associated degradation (ERAD) and consequently ER stress, which in turn activates apoptosis pathways (32). By blocking these pathways for ER stress-mediated apoptosis, Parkin is able to improve cell viability (32). Thus, alongside the proteasomal degradation, ataxin-3 seems to also be involved in the regulation of misfolded ER protein degradation. In fact, Ataxin-3 has been found to bind to valosin-containg protein(VCP) forming a VCP/ataxin-3 complex that seems to also be involved in regulating ERAD and assisting in targeting proteins to the proteasome (25,33). This VCP/ataxin-3 complex, e.g., possibly associated with E3 ligase E4B, appears to regulate the ubiquitination status and subsequent degradation of insulin/insulin-like growth factor 1 (IGF1) signaling pathway elements, a pathway that has been implicated in lifespan regulation (25).

Mytochondrial dysfunction and impaired energy metabolism

Neurodegeneration has been correlated with mitochondrial dysfunction and impaired energy metabolism (34). In fact, several lines of evidence have implicated mitochondrial dysfunction as a mechanism of neurodegeneration in polyQ diseases as in several other neurodegenerative diseases, such as Alzheimer's disease, PD and amyotrophic lateral sclerosis (ALS) (34).
Several reports have demonstrated that expanded polyQ proteins accumulate not only in the nucleus but in the mitochondria as well, leading to mitochondrial dysfunction and subsequent cell death (34-38). Mitochondria is well known to play a crucial role in several cellular events such as ATP synthesis by oxidative phosphorylation, Iron and lipid metabolism, calcium buffering and cell death (34).
A mitochondrial ubiquitin ligase, MITOL, localized in the mitochondrial outer membrane, has caught the attention of scientists for its apparent protective role in controlling mitochondrial polyQ aggregates formation and toxicity (34). Results indicate that MITOL directly ubiquitinates and promotes degradation of mitochondrial, but not cytosolic, pathogenic ataxin-3 via UPS, attenuating its toxicity (34). Thereby, because of its demonstrated protective role against polyQ toxicity, MITOL has been found to be a potential new therapeutic target in polyQ diseases (34).
As mentioned previously, oxidative stress was found to contribute to MJD pathogenesis as well as to other late-onset neurodegenerative disorders. The brain has one of the highest oxygen consumption metabolic rates of the body and oxidative stress can be expected to have deleterious effects on neuronal function (35). Oxidative stress, induced by reactive oxygen species (ROS) or free radicals, increases with age and it has been suggested that cells throughout the time decrease on their capacity to protect against oxidative stress accumulated with time. This seems to happen through a decline on their antioxidant defenses, resulting in an imbalanced oxidant/antioxidant ratio (36). Non-enzymatic and enzymatic components play critical roles in the cellular defense system against oxidative stress in the brain (36). The antioxidant enzymes known to be included in this system are catalase (CAT), glutathione disulfide reductase (GSSG-R), superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) and the non-enzymatic components that seem to play central roles in removing the excess of H2O2 in the brain are glutathione (GSH) and ascorbate (36). In a study conducted with SK-N-SH

and COS-7 cell lines overexpressing either normal and expanded ataxin-3 (Q78), results suggested that in cells harboring the expanded ataxin-3, the activities of GSSG-R, CAT and SOD were significantly reduced, whereas the GSH-px enzyme activity did not change significantly compared to control cells (36). This decrease on enzymatic activity is probably responsible for the decrease amount of GSH and total glutathione content and consequently low level of GSH/GSSG ratio found in mutant MJD cells. Thus, a lowered enzymatic components activity in the presence of expanded ataxin-3 possibly leads to a decrease on the antioxidant defense effectiveness in cells expressing the mutant protein (36). Furthermore, mitochondrial DNA (mtDNA) under oxidative stress is known to be susceptible to damage, as it was demonstrated in mutant cells and SCA3 patient blood samples, the mitochondrial DNA copy numbers were reduced when compared to normal controls. One crucial factor that mediates neurons survival is the maintenance of mtDNA copy number hence its decrease possibly contributes to the progression of neurodegenerative disease (36).

A major independent risk factor for progressive neurodegenerative disorders is ageing and mitochondrial dysfunction has also been implicated in ageing, as it plays key roles in regulating cellular survival and apoptotic death (36). In a study conducted in CNS neurons expressing polyQ expanded ataxin-3 (Q79) with the aid of recombinant adenoviruses, results provided evidence that mutant ataxin-3 induces apoptotic cell death by promoting mitochondrial release of apoptogenic proteins, cytochrome c and second mitochondria-derived activator of caspase (Smac) (37). This release is preceded by the upregulation of Bax protein, a proapoptotic protein, and downregulation of Bcl-xL expression, an anti-apoptotic protein (37). Bcl-2 family of proteins is a group of key regulators of the mitochondrial apoptotic pathway. This family of proteins includes antiapoptotic Bcl-2 and Bcl-xL and proapoptotic Bak and Bax (37). As mentioned before, mutant polyQ proteins induce transcriptional dysregulation, a step that seems to be critical in the pathogenesis of polyQ diseases. Therefore, the upregulation of Bax and downregulation of Bcl-xL protein seems to likely result from the alteration of the respective mRNA expression (37). Accordingly, Bax mRNA levels were found significantly increased in cultural neurons expressing mutant ataxin-3 (Q79), in opposition to the levels of Bcl-xL mRNA, which were found downregulated (37). Thus, mutant ataxin-3 (Q79) induced an increased on Bax/Bcl-xL ratio protein expression, likely promoting further activation of Bax and Bak, which in turn induced permeabilization of mitochondrial outer membrane, promoting the efflux of cytochrome c and Smac to the cytosol (37). As a result, this redistribution of cytochrome c and Smac to the cytosol was demonstrated to lead to the activation of caspase-9 and caspase-3, the latter via caspase-9-mediated mitochondrial apoptotic pathway (37). As previously mentioned, caspases are cysteine proteases associated with apoptotic pathways and their activation can follow two pathways: one where the cytochrome c and Smac release from the mitochondria to cytosol induces caspase activation, the first by forming a complex with Apaf-1 and inducing cleavage and activation of procaspase-9, the second (Smac) by suppressing the effect of IAP family of proteins that inhibit caspase activity; the other pathway results from the activation of membrane death receptors that induce autocatalytic activation of procaspase-8 by recruiting an adaptor protein called FADD. Once

the respective caspases are activated they initiate the apoptotic process by activating downstream effector caspases (37).
In addition, mitochondrial complex II was found decreased in MJD transgenic mouse models and lymphoblast cell lines derived from MJD patients (38). Mitochondrial complex II is the only mitochondrial complex of the respiratory chain that is exclusively nuclear-encoded and impairment of this complex might be essential to some of the biochemical changes elicited by various polyQ expanded proteins, leading to a further increase oxidative stress in the cell (38).

Emerging therapeutic Strategies

Understanding the mechanisms and the key molecular players involved in neurodegenerative diseases is central for the development of effective therapeutic approaches and major research advances have been made in this area (1,21). Even though new therapeutic strategies are being devised, only symptomatic treatment is available for polyQ diseases. For instance, for MJD, the pharmacological therapies available seek to reduce symptomatic features such as depression, Parkinsonian signs, spasticity, restless leg syndrome and the aberrant sleep patterns experienced by many MJD patients (1,21,33). In addition, physiotherapy and regular speech therapy can assist patients to cope with disease-associated disability mainly regarding gait symptoms, dysarthria and dysphagia, respectively (1).
As mentioned previously regarding proteolytic processing, inhibition of caspases and calpains have been shown to be capable of slowing disease progression and reducing the toxicity of the mutant protein (8, 10, 13). However, caspase functions in the brain are complex and mediate important functions for the normal brain, such as apoptosis, dendritic development, synaptic plasticity, hence the use of caspase inhibitors appears to be improbable as a therapeutic strategy. Regarding calpains, there are several types of calpain inhibitors available, but the lack of specificity amongst calpain isoforms and other proteolytic enzymes discourages its use as a therapeutic tool (33).
Reducing the aggregation and toxicity of expanded polyQ proteins has been considered a therapeutic approach, however no promising candidates for application in the clinic have yet been identified (33). Some studies have suggested that increasing chaperone activity (e.g. Hsp 40 and Hsp70) may prove beneficial in polyQ diseases, since overexpression of selective Hsp chaperones were found to reduce polyQ proteins aggregation and toxicity by aiding in protein refolding and degradation (39). However, pharmacological induction of molecular chaperone (e.g. geldanamycin and geranylgeranyl acetone) or designed chemical chaperones (trimethylamine N-oxide, glycerol, dimethyl sulfoxide) were found to be limiting for clinical use given the side effects and cytotoxicity at concentrations required for the expected outcome (9, 33).
As for modulating calcium homeostasis that, as mentioned before, has been described to be dysregulated in polyQ diseases, Dantrolene was found to inhibit excessive calcium release in

transgenic MJD mice, improving motor performance and preventing neuronal loss with no reported adverse events (33).

Transcriptional dysregulation is thought to play a role in the pathogenesis of polyQ diseases. Mutant ataxin-3 appears to disrupt the activity of key factors in transcription, some of which retain acetyltransferase activity (9, 40). Sodium butyrate (SB), a histone deacetylase (HDAC) inhibitor, was shown to reverse the mutant ataxin-3 hypoacetylation and transcriptional repressor activity, ameliorating the motor impairment seen in SCA3 transgenic mice (9, 33). However, these beneficial neuroprotective effects were yielded within a narrow therapeutic window (40). Valproic acid (VPA) is another HDAC inhibitor that has been established as clinically safe and tolerable (40). VPA has been demonstrated to induce apoptosis-inhibiting gene bcl-2 thereby mediating neuronal protection and in MJD *Drosophila* model it was found to alleviate polyQ-induced phenotypic abnormalities (40). Results suggest that HDAC inhibitors are potential therapeutic agents for MJD and other polyQ diseases (40).

As previously mentioned, upregulation of autophagy and clearance mechanism through the UPS is an appealing strategy to alter the progression of MJD. Overexpression of beclin-1 was found to be able to rescue or slow down the progression of MJD ataxic phenotype, depending on the stage of disease at the time of intervention (26,27). Upregulation of autophagy by rapamycins and temsirolimus, a rapamycin ester, was also found to reduce toxicity of aggregate-prone proteins by mechanisms described earlier (28). In addition, lithium, which is commonly used to treat bipolar disorders, was also shown to be able to upregulate autophagy in an mTOR independent manner, having beneficial effects in polyQ diseases, including MJD, HD as well as in ALS patients and mouse models, where it was shown to delay disease progression by facilitating superoxide dismutase 1 clearance (28, 33).

As oxidative stress has been found to contribute to MJD pathogenesis, antioxidant-based therapies, although scarcely researched, could be able to provide some neuroprotective effects. Coenzyme Q10 possesses antioxidant potential when reduced to ubiquinol and it has been found to have some beneficial effects in HD, currently being tested in clinical trials of HD (33).

A promising therapeutic approach in monogenetic disorders such as MJD, where the mutant allele acts through a dominant toxic mechanism, is to turn off the responsible gene, suppressing its expression and mitigating disease pathogenesis. This gene silencing strategy has been successfully used in rodent models of several autosomal dominant neurodegenerative diseases, including HD, ALS and SCA1, delaying disease onset progression and protecting against neurodegeneration (33, 41, 42). Lacking discrimination between normal and mutant forms of the protein can represent a potential risk of gene silencing by nullifying the normal protein function (33,42). Since, ataxin-3 has been implicated in protein quality control pathways and its normal activity was found to have a protective role and to mitigate neurodegeneration in *Drosophila* models (6), silencing the normal protein might lead to impair neuronal function and cell viability (33, 41, 42). In order to spare normal ataxin-3 function, allele-specific downregulation of mutant ataxin-3 has been researched (33,41-43). Two strategies for allele-specific downregulation have been postulated. The first strategy approach is

based on the presence of a single nucleotide polymorphism (SNP) identified at the 3'end of CAG tract of ATXN3 gene that was found to be present in 70% of MJD patients, but in only 2% of the normal population (41,42). Using lentiviral vectors encoding short-hairpin RNAs (shRNA) targeting the specific intragenic SNP of the mutant ataxin-3 gene, a report was able to demonstrate *in vivo* downregulation of mutant ataxin-3 in a selective manner, associated with a significant decrease in the formation and accumulation of inclusions, reduced number of degenerating neurons and neuronal dysfunction (42). In a subsequent work, it was also shown in transgenic MJD mouse models, exhibiting an early and severe phenotype, that allele-specific silencing of mutant ataxin-3 improves motor coordination and alleviation of neuropathology even when initiated at late stages of disease (41). However, this approach is limited to MJD patients carrying the heterozygous SNP in the ATXN3 gene (43) and there's still important questions that will first need to be addressed before a similar strategy can be attempted in a clinical trial for MJD, such as finding a suitable delivery of shRNAs to precise, targeted brain regions as determining the safety of a long-term shRNAs treatment (33, 41).
The second strategy approach is to target directly the expanded CAG repeat, removing it from the mutant ataxin-3 protein, reducing its toxicity through protein modification (43).
Through the use of small molecules, such as antisense oligonucleotides (AON) or peptide nucleic acids (PNAs), a study was able to achieve allele-specific reduction of mutant ataxin-3 by using AONs to mask exons of the ataxin-3 pre-mRNA from the splicing machinery, consequently excluding the targeted exon from subsequent translation leading to the formation of a modified ataxin-3 protein, lacking the polyQ stretch but maintaining its ubiquitin binding properties and functional domains intact (43). In the respective study, to preserve the reading frame for subsequent translation exon 9 and exon 10 needed to be skipped simultaneously (43). The CAG repeat in ATXN3 gene is located in exon 10, whereas exon 9 seems to encode a calcium-dependent calpain cleavage and a NLS. There were no observed toxic properties from the resulting truncated ataxin-3 protein and the overall ataxin-3 transcript and protein levels were preserved (43). Another advantage of this AON-based protein modification is that is can be applied to all MJD patients, in contrast to SNP-specific approach (43). These results represent a promising approach, although there is still some concerns regarding their specificity (33) and there are still questions that remain to be assessed about the resulting modified protein, e.g., the impact of exon skipping in localization, function and aggregation (43).
In a more recent study conducted in MJD mice models, transplantation of cerebellar neural stem cells (NSC) into the cerebellum of diseased mice led to a significant and ample reduction in MJD-associated neuropathology, alleviating and improving the motor phenotype, promoting neuroprotection by increasing the levels of neurotrophic factors, such as BDNF and modulating neuroinflammation, reducing the levels of proinflammatory mediators, such as IL1β and TNFA (44). Thus, cerebellar NSC transplantation has demonstrated that cell replacement might be an effective treatment in symptomatic patients with extensive neuronal loss (44).

Concluding Remarks

Several pathways have been targeted in the quest for a therapy for MJD, but an effective treatment is still currently lacking and of the therapeutic strategies in play none has yet advanced to human clinical trials. Even so, these strategies hold great promise as therapeutic approaches and as potential routes towards a therapy for MJD, either by targeting the expression, processing or conformation of the mutant protein or by reversing the cellular defects.

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SINAPSE

A SINAPSE é uma revista médica, propriedade da Sociedade Portuguesa de Neurologia (SPN), publicada em edição clássica e em suporte electrónico.

A SINAPSE é orgão oficial da Sociedade Portuguesa de Neurologia (SPN), incluindo as secções e os grupos de estudos, da Liga Portuguesa Contra a Epilepsia (LPCE), da Sociedade Portuguesa de Cefaleias (SPC), da Sociedade Portuguesa de Estudos de Doenças Neuromusculares (SPEDNM) e da Sociedade Portuguesa de Neuropatologia (SPNp).

Princípios gerais

A SINAPSE orienta-se pelos seguintes princípios gerais:

1. Defesa e promoção da Neurologia Clínica portuguesa;
2. Apoio empenhado e independente às iniciativas de SPN, LPCE, SPC, SPEDNM, SPNp, espelhando os seus estadios de desenvolvimento e contribuindo para a sua consolidação e robustez;
3. Prática da Neurologia Clínica como vocação primordial;
4. Trabalhos transversais, integradores ou promotores da unidade da Neurologia como interesses privilegiados;
5. Preservação da memória das instituições como preocupação permanente;
6. Especialidades médicas afins e neurociências como interesses potenciais;
7. Abertura e acessibilidade a pessoas e a instituições;
8. Procura de qualidade técnico-científica, formal e estética;
9. Rigor e pedagogia na aplicação sistemática das normas do "ICJME-International Committee of Medical Journal Editors" (http://www.icmje.org);
10. Garantia de independência científica e editorial, relativamente aos Órgãos Sociais da SPN, patrocinadores ou outras entidades;
11. Predisposição para a mudança.

Órgãos da SINAPSE

1. **Administração**. É composta por três elementos da Direcção da SPN (Presidente, Vice-Presidente para a área editorial e Tesoureiro), sendo responsável pelas componentes económicas, financeiras e logísticas.
2. **Director.** É nomeado pela Direcção da SPN, podendo ser membro dos Órgãos Sociais ou independente; estabelece a orientação global, a preparação e execução das edições, ouvido o Conselho Editorial.
3. **Conselho Editorial.** É nomeado pela Direcção da SPN, mediante proposta fundamentada do Director, sendo os seus membros sócios independentes dos Órgãos Sociais; compete ao Conselho Editorial participar nas grandes opções de natureza editorial, científica e estética.
4. **Conselho Científico.** É, por inerência, o Conselho Científico da SPN, competindo-lhe garantir o rigor ético e técnico-científico das publicações.

Normas de candidatura

1. Os trabalhos candidatos a publicação serão inéditos, e não deverão ser enviados para outras publicações.
2. Deverão ser remetidos por correio electrónico, em documentos anexos (*attached files*) Microsoft Word™, em qualquer versão actual.
3. Deverão ser evitados símbolos, sublinhados, palavras em maiúsculas, *bolds*, itálicos, notas de topo ou de rodapé, e artifícios formais.
4. As páginas não deverão ser numeradas.
5. Deverão ser redigidos em português ou em inglês. Poderão, excepcionalmente, aceitar-se trabalhos em francês ou espanhol.
6. Da primeira página constarão: título do trabalho, nome próprio, apelido, departamento ou serviço, instituição, profissão, cargo, endereço, telemóvel e correio electrónico de todos os autores.
7. A segunda página incluirá: o título do trabalho, o nome dos autores, o resumo, as palavras-chave e o título de cabeçalho; a morada institucional e o endereço de correio electrónico a incorporar no artigo.
8. A terceira página será a versão em inglês da segunda página, se o artigo foi redigido em português (e vice-versa). Se o artigo for redigido em francês ou espanhol, a terceira e quarta página serão versões em português e Inglês, respectivamente.
9. As restantes folhas incluirão as diferentes secções do trabalho. Os trabalhos originais incluirão as seguintes secções: introdução/objectivos, metodologia, resultados, discussão/conclusões e bibliografia. Os casos clínicos serão estruturados em introdução, caso clínico, discussão e bibliografia. As revisões incluirão, pelo menos, introdução, desenvolvimento, conclusões e bibliografia. Os editoriais e as cartas estarão isentos de organização em secções. No texto das secções, a identificação institucional será evitada, podendo ser acrescentada, se imprescindível, no fim do processo de avaliação e antes da publicação do artigo.
10. As tabelas e figuras deverão ser enviadas em documento adicional Microsoft Word™, uma por página, precedidas por uma página que inclua as notas correspondentes. As figuras serão enviadas em ficheiros GIF ou JPEG.
11. Os agradecimentos ou menções particulares constarão em página própria.
12. Os compromissos particulares ou institucionais (patrocínios, financiamentos, bolsas, prémios) serão expressos obrigatoriamente em página adicional.

Regras para elaboração do trabalho

1. Título

Será claro e informativo, representativo do conteúdo do artigo e captando a atenção do leitor. Não terá iniciais ou siglas, nem excederá vinte palavras. Sub-títulos genéricos ou vulgares como “caso clínico” ou “ a propósito de um caso clínico” não serão aceites.

2. Autores e instituições

A autoria exige, cumulativamente, contribuições substanciais para:

a) concepção e desenho, ou aquisição de dados, ou análise e interpretação de dados;

b) redacção ou revisão crítica de uma parte importante do seu conteúdo intelectual;

c) responsabilidade pela aprovação da versão final.

Cada um dos autores deve ter participado suficientemente no trabalho para assumir responsabilidade pública pelo seu conteúdo.

A obtenção de financiamento, a colecção de dados ou a supervisão da equipa de investigação não justificam a autoria.

Todas pessoas designadas por autores devem cumprir os critérios; nenhuma pessoa qualificada para autoria deve ser excluída.

Membros do grupo de trabalho (coordenadores, directores, técnicos, consultores), que não cumpram os critérios internacionais de autoria, poderão ser listados em “agradecimentos”.

O número de autores será parcimonioso, particularmente em “Casos Clínicos”.

A inclusão e compromisso do nome das instituições é da responsabilidade dos autores.

3. Resumo

O resumo tem um limite máximo de 400 palavras. Não deve incluir abreviaturas. Deve apresentar-se estruturado.

Originais: Introdução, Objectivos, Metodologia, Resultados e Conclusões.

Revisões: Introdução, Objectivos, Desenvolvimento e Conclusões.

Casos clínicos: Introdução, Caso Clínico e Conclusões.

O resumo será coerente com o conjunto doo artigo.

4. Palavras-chave

Devem ser incluídas até seis palavras-chave, na língua original do artigo e em inglês, preferencialmente previstas na lista do *Medical Subject Headling List of the Index Medicus.*

5. Cabeçalho

Versão reduzida do título, para eventuais efeitos de composição gráfica.

6. Introdução / Objectivos

Exposição, completa e sucinta, do estado actual do conhecimento sobre o tema do artigo.

Expressão clara das motivações e objectivos que levaram ao planeamento do trabalho.

7. Metodologia

Descrever os critérios de selecção do material do estudo e o desenho do mesmo.

Usar unidades internacionais.

Assinalar os métodos estatísticos.

8. Resultados

Devem ser escritos os dados relevantes.

Os dados constantes de tabelas ou figuras não devem, em princípio, ser repetidos no texto.

As tabelas devem ser nomeadas em numeração romana (p. ex.: Tabela IV), por ordem de aparecimento no texto.

As figuras devem ser nomeadas em numeração árabe (p. ex.: Fig. 4.), pela ordem de aparecimento no texto.

A responsabilidade de protecção dos direitos de figuras previamente publicadas é da responsabilidade dos autores.

A publicação de fotografias de pessoas exige a completa dissimulação da sua identidade ou uma folha assinada de consentimento informado e parecer de uma Comissão de Ética de uma instituição pública..

9. Discussão

Não voltar a apresentar resultados, evitando redundâncias.

Não mencionar dados que não foram apresentados nos resultados.

Dar-se-á relevo aos aspectos novos, reflectir sobre as limitações e justificar os erros ou omissões.

Relacionar os resultados com outros estudos relevantes.

As conclusões deverão basear-se apenas nos resultados.

Poderão fazer-se recomendações.

10. Bibliografia

As referências bibliográficas devem ser identificadas no texto através de numeração árabe, entre parêntesis, ao nível da linha.

Devem ser numeradas segundo a ordem de aparecimento no texto.

A referência deve incluir o apelido e inicial de todos os autores; se o artigo tiver mais de seis autores, devem ser referidos apenas os três primeiros, seguindo-se a expressão *et al*.

Os nomes dos autores devem ser seguidos por título do artigo, abreviatura da revista *segundo as* recomendações do *List of Journals Indexed in Index Medicus*, ano de edição, volume, primeira e última página.

As referências a livros devem incluir o título do livro, seguido do local de publicação, editor, ano, e páginas relevantes.

Se alguma referência se encontrar pendente de publicação deverá descrever-se como "in press". A referência a comunicações pessoais não é aceitável.

11. Dúvidas ou casos omissos

Serão resolvidos de acordo com as normas do ICMJE (http://www.icmje.org).

Processo de Avaliação e Edição

1. A SINAPSE notificará o primeiro autor, imediatamente após a recepção do trabalho;
2. A SINAPSE poderá devolver imediatamente o trabalho aos autores para correcções formais, de acordo com as normas de publicação;
3. Após recepção definitiva, o trabalho será enviado a todos os membros do Conselho Editorial, que sugerem os revisores adequados (membros do Conselho Editorial, do Conselho Científico ou independentes). Os revisores não terão ligações às instituições constantes do trabalho. Os membros do Conselho Editorial e os revisores não serão informados dos nomes e instituições dos autores;
4. Os autores terão acesso aos pareceres anónimos dos revisores;
5. Os autores terão quinze dias úteis para alterar o artigo e/ou contestar as revisões;
6. As respostas serão analisadas pelo Conselho Editorial, podendo ser remetidas aos revisores para novo parecer;
7. A Direcção da SINAPSE assumirá a aceitação ou rejeição do trabalho para publicação, após análise e interpretação final de todos os documentos;
8. Os autores terão acesso aos conjunto dos documentos, em caso de rejeição do trabalho, mantendo-se oculta a identidade dos revisores.

Os trabalhos aceites serão publicados na edição seguinte da SINAPSE, após assinatura de uma norma de responsabilidade e transferência de direitos por todos os autores. Por critérios editoriais, a Direcção da SINAPSE poderá acordar com os autores o adiamento da publicação.