

# Discovery of Therapeutic Approaches for Polyglutamine Diseases: A Summary of Recent Efforts

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Published online in Wiley Online Library (wileyonlinelibrary.com).

DOI 10.1002/med.21425



**Abstract:** Polyglutamine (PolyQ) diseases are a group of neurodegenerative disorders caused by the expansion of cytosine–adenine–guanine (CAG) trinucleotide repeats in the coding region of specific genes. This leads to the production of pathogenic proteins containing critically expanded tracts of glutamines. Although polyQ diseases are individually rare, the fact that these nine diseases are irreversibly progressive over 10 to 30 years, severely impairing and ultimately fatal, usually implicating the full-time patient support by a caregiver for long time periods, makes their economic and social impact quite significant. This has led several researchers worldwide to investigate the pathogenic mechanism(s) and therapeutic strategies for polyQ diseases. Although research in the field has grown notably in the last decades, we are still far from having an effective treatment to offer patients, and the decision of which compounds should be translated to the clinics may be very challenging. In this review, we provide a comprehensive and critical overview of the most recent drug discovery efforts in the field of polyQ diseases, including the most relevant findings emerging from two different types of approaches—hypothesis-based candidate molecule testing and hypothesis-free unbiased drug screenings. We hereby summarize and reflect on the preclinical studies as well as all the clinical trials performed to date, aiming to provide a useful framework for increasingly successful future drug discovery and development efforts. © 2016 Wiley Periodicals, Inc.

Med. Res. Rev., 00, No. 0, 1–47, 2016

**Key words:** polyglutamine diseases; therapeutic strategies; preclinical trials; clinical trials

## 1. INTRODUCTION

Polyglutamine (PolyQ) diseases are a large group of inherited neurodegenerative disorders characterized by the pathological expansion of cytosine–adenine–guanine (CAG) repeats in the coding sequences of distinct genes leading to the production of mutant proteins bearing expanded polyQ tracts. PolyQ diseases include Machado–Joseph disease (MJD/SCA3),<sup>1</sup>

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Medicinal Research Reviews, 00, No. 0, 1–47, 2016

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Huntington's disease (HD),<sup>2</sup> spinal and bulbar muscular atrophy (SBMA),<sup>3</sup> dentatorubral-pallidoluysian atrophy (DRPLA),<sup>4,5</sup> and other spinocerebellar ataxias (SCA1, SCA2, SCA7, and SCA17<sup>6-12</sup>; see Table I for a summary of disease features). The frequency of polyQ diseases averages 1–10:100,000<sup>13</sup>; of these, HD and MJD/SCA3 are the most common worldwide.<sup>14</sup> All are progressive, ultimately fatal disorders that typically begin in midlife and progress over 10 to 30 years. PolyQ diseases are inherited in an autosomal dominant manner, with the exception of SBMA, which is X-linked, and they only manifest when the glutamine expansions exceed a certain threshold.<sup>15</sup> Above the pathogenic threshold, the CAG tracts are usually unstable across generations, and they can expand or contract in a variably manner, particularly depending on the sex of the transmitting parent.<sup>16</sup> This intergenerational instability causes, on average, an increase in repeat length, leading to earlier age-at-onset and increased severity of the symptoms in the successive generations, a phenomenon known as anticipation.<sup>17-19</sup> In addition to intergenerational instability, tissue-specific mosaicism has been observed in polyQ diseases, resulting in cell populations carrying different CAG repeat lengths, within or between tissues.<sup>20-24</sup> This CAG repeat instability underlies some features of polyQ disorders and may also contribute to their pathogenic mechanism.<sup>25-27</sup>

At the molecular level, the disease-causing proteins are ubiquitously expressed, in the nervous system and peripheral tissues; however, each polyQ disease is characterized by the loss of specific neuronal populations, resulting in typical patterns of neurodegeneration and characteristic clinical symptoms.<sup>28</sup> A common feature of all polyQ disorders is the accumulation of aggregated forms of the mutant protein, in the cytoplasm and mostly in the nucleus of the neuronal cells, ultimately forming neuronal intranuclear inclusions (NIIs), which are found in patient brains and transgenic models of the disorders.<sup>29,30</sup> These inclusions are a common hallmark of all these diseases; however, it is still controversial if they are pathogenic or not.<sup>31-34</sup> Learning from early investigations in Alzheimer's disease (AD) and Parkinson's disease (PD) studies (reviewed in 35), several studies have explored which type of structure formed by the expanded polyQ is responsible for the disease (reviewed in 36 and discussed in Section 2.1.3). On the one hand, in the initial steps of aggregation, some of the monomeric species that transform into oligomers and protofibrils/fibrils may be direct intermediates for end-stage aggregate formation, while others may not be,<sup>37</sup> and it is very important to define each of these groups. On the other hand, the intermediate species along the aggregation pathway may constitute the most toxic insult to the cell or, at least, have a considerable contribution for pathogenesis. In fact, both in AD and PD, current research indicates that oligomeric forms are probably the key pathogenic elements (reviewed in 35): large inclusions are increasingly suggested not to be so highly toxic,<sup>38,39</sup> whereas smaller  $A\beta$  and  $\alpha$ -synuclein aggregates and intermediate species such as the oligomers are thought to be the toxic species.<sup>40,41</sup> Although this is less clearly defined for polyQ diseases and the exact role of polyQ aggregation has been widely discussed, one idea that seems consensual is that (like in AD) cleaved versions of the mutant proteins are more toxic than their full-length counterparts, that is, proteolytic cleavage of the original proteins leads to the formation of fragments that contain the polyQ tract, which are more prone to aggregation, and seem to constitute a more toxic insult to the cell. This is known as the *toxic fragment hypothesis*, and the presence of such fragments was shown in several polyQ diseases.<sup>42-51</sup>

The theory that a toxic structure of the expanded polyQ causes the disease explains its length dependence (specific for each polyQ disease, but within very similar thresholds), and implies a common pathogenic mechanism in these diseases. Although this hypothesized common mechanism is not sufficient to explain the selective toxicity pattern of each of these causative proteins, which leads to the slightly different neuropathologic affection and clinical manifestations of each disease,<sup>52</sup> its clarification is expected to lead to therapeutic approaches that could be of use for many of these diseases.

**Table 1.** PolyQ Diseases, Causative Genes, Proteins, Brain Regions Affected, and Main Clinical Features

PolyQ disease	Locus	Protein	Protein localization	CAG repeat size		Affected brain regions	Clinical features
				Normal	Pathological		
HD	4p16.3	Huntingtin	Cytoplasmic	6–35	36–121	Striatum and cerebral cortex	Chorea, dystonia, dementia
SBMA	Xq11-q12	Androgen receptor	Nuclear and cytoplasmic	6–36	38–62	Anterior horn, bulbar neurons and dorsal root ganglia	Weakness, bulbar symptom, fasciculations, tremors, gynecomastia
DRPLA	12p13	Atrophin-1	Cytoplasmic	3–38	49–88	Pallidum, subthalamic, dentate nuclei and red nucleus	Ataxia, chorea, dementia and myoclonus epilepsy
SCA1	6p23	Ataxin-1	Nuclear	6–39	41–83	Cerebellar Purkinje cells, dentate nucleus, brainstem	Ataxia, bulbar symptoms, spasticity, neuropathy, cognitive impairment
SCA2	12q24	Ataxin-2	Nuclear and cytoplasmic	14–32	34–77	Cerebellar Purkinje cells, brainstem, frontotemporal lobes	Ataxia, slow saccades, polyneuropathy, parkinsonism
MJD/SCA3	14q24-q31	Ataxin-3	Nuclear and cytoplasmic	12–40	62–86	Deep cerebellar nuclei, brainstem, basal ganglia, subthalamic nuclei, red nucleus, spinal cord	Ataxia, spasticity, dysphagia, ophthalmoplegia, dystonia, rigidity, frequent urination
SCA6	19p13	CACNA1A	Cytoplasmic	4–18	21–30	Cerebellar Purkinje cells, dentate nucleus, inferior olive	Ataxia, dysarthria, down-beating nystagmus
SCA7	3p21-p12	Ataxin-7	Nuclear	7–18	38–200	Cerebellum, brainstem, macula, visual cortex	Ataxia, retinal degeneration, ophthalmoplegia.
SCA17	6q27	TBP	Nuclear	25–43	45–63	Cerebellar Purkinje cells, inferior olive	Ataxia, dementia, psychosis, seizures, extrapyramidal signs

Source: Adapted from 3,6,10, and 79-84.

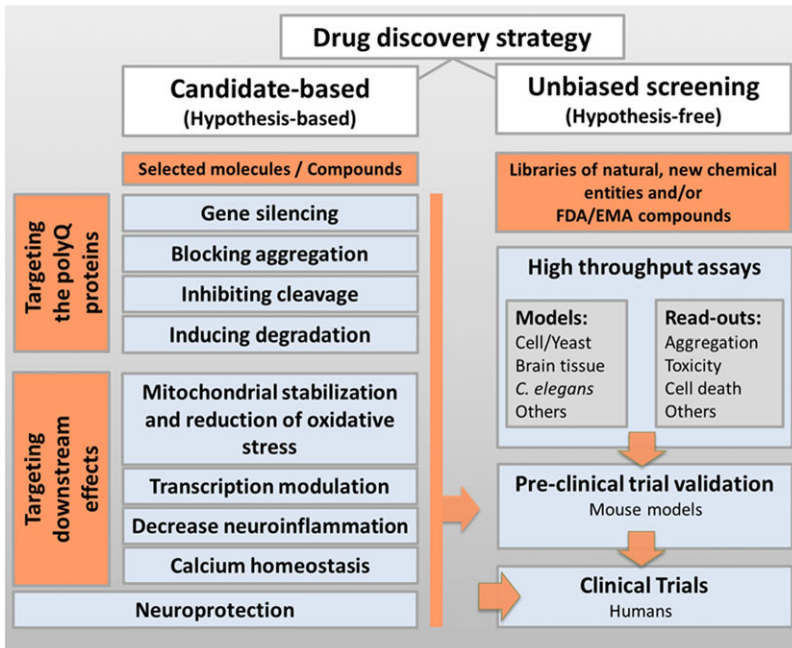
Other hypotheses have been put forward for polyQ pathogenesis, mainly the evidence for the cell-to-cell propagation of the pathogenic proteins that form the hallmark lesions of these neurodegenerative disorders.<sup>53</sup> In this hypothesis, the protein aggregates can in some cases become infectious and act similarly to prion disorders. The majority of the studies were performed in HD and suggested that aggregates can replicate, like prions, being able to cross cellular membranes and “infect” neighbor cells, thus contributing to the disease progression.<sup>54–56</sup> Several studies have addressed the underlying mechanisms of cell-to-cell aggregate transmission, release, and uptake.<sup>53, 57, 58</sup>

In these diseases, neurons are more frequently affected because they are probably more sensitive to mutant proteins than other cell types and, since they do not divide, mutant polyQ proteins more quickly accumulate in these cells. Clearance of misfolded and mutant proteins is mostly accomplished by the Ubiquitin Proteasome System (UPS) and even more so by autophagy, processes of high relevance in neurons and whose activities appear to decrease during the course of the disease.<sup>59–63</sup> Nevertheless, mutant proteins are generally ubiquitously expressed throughout the body and affected peripheral cells might also contribute to the disease process.<sup>64, 65</sup> Several studies, mostly in HD, have been carried out to unravel the contribution of peripheral cells to the disease,<sup>66–71</sup> but this still needs to be further assessed, and efforts are still lacking to unravel whether these changes are primarily caused by the expression of mutant polyQ proteins in peripheral tissues or are secondary effects of nervous system dysfunction.

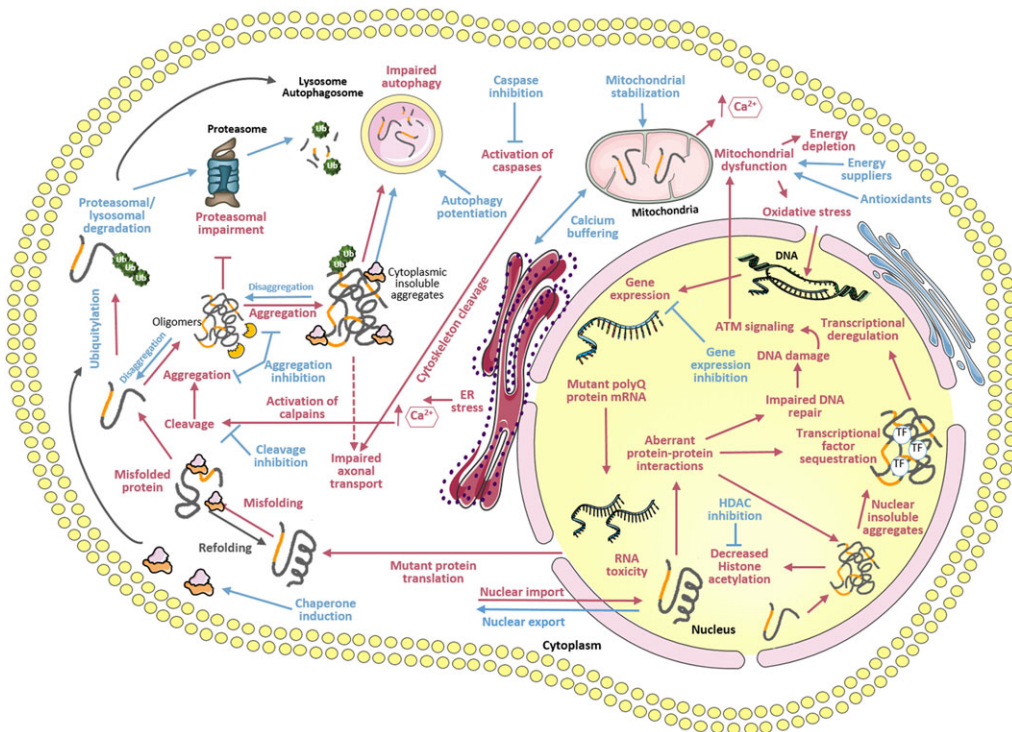
Despite the ubiquitous expression of polyQ-proteins, cell-specific neurodegeneration does exist in this group of diseases. The consistent loss of these neuronal populations has been proposed to result from the differential vulnerability of these cells to the toxic gain of function associated with polyQ expansion. As an example, the vulnerability of the medium spiny neurons (MSN) in the striatum and the pyramidal neurons in the cortex of HD patients has been related to their morphology, neurotransmitter content, and electrophysiological properties (reviewed in 72). The specific interactions of a given polyQ protein with other tissue- or cell-specific proteins could also contribute to explain the specificity of neuronal death. For example, in SCA7 it was demonstrated that retinal degeneration could be associated with an altered function of cone-rod homeobox protein CRX,<sup>73</sup> whereas in HD the striatum-enriched huntingtin interactor Rhes has been shown to be key for the selective toxicity of this polyQ protein.<sup>74–78</sup> An understanding of the interactions between polyQ-proteins and cell type-specific partners and cellular features may provide a novel conceptual framework for the development of effective therapeutic strategies in polyQ disorders.

## **2. THERAPEUTIC STRATEGIES FOR POLYQ DISEASES**

Despite great progress in elucidating the pathogenic mechanisms underlying polyQ diseases, an effective treatment is still lacking. The current strategies toward discovery and development of therapies for these diseases can be divided according to the model of choice of molecules to be tested, in candidate-based (or hypothesis-based) studies and unbiased (hypothesis-free) screenings. The most common are those based on molecules with known properties, targeting specific aspects of the hypothesized pathological mechanisms, which (see Fig. 1) can further be divided in two categories: (i) those targeting the expression, processing, or conformation of the causative polyQ protein; (ii) those preventing the toxic effects of the polyQ protein, such as mitochondrial dysfunction and oxidative stress, transcriptional dysregulation, UPS and autophagy impairment, excitotoxicity, and/or activation of cell death pathways (see Fig. 2 for a summary of pathogenic mechanisms and current candidate-based therapeutic approaches); and, lastly, (iii) those providing a more general neuroprotection.<sup>85</sup> On the other hand, unbiased drug screenings are gaining relevance mostly through the analysis of large libraries of



**Figure 1.** Workflow of drug discovery strategies for polyQ diseases considering the selection of molecules to test (hypothesis-based or hypothesis-free/unbiased) and the level of mechanistic targeting (the polyQ proteins themselves or the downstream cellular effects). Unbiased screenings can be based on different read-outs, such as aggregation or toxicity, among others, used to measure efficacy of the compounds.



**Figure 2.** Pathogenic mechanisms of polyQ diseases and current hypothesis-based therapeutic approaches.

well-known or new compounds with high-throughput methodologies. These approaches often reveal interesting disease-modifying agents, contributing not only to define novel elements within mechanisms of the disease, but also, particularly in the case of repurposing screens, allowing for a direct pathway to therapy.

Given that neurodegenerative disorders share many features, it is not surprising that the field PolyQ diseases benefit from the knowledge accumulated in other (significantly more common) diseases, not only in terms of their pathogenic mechanisms but also regarding therapeutic approaches. Several studies in AD, PD, or amyotrophic lateral sclerosis (ALS) are in fact used to direct the therapeutic strategies for PolyQ diseases, as referred along this review. The reverse should also not be discouraged, since recent findings in PolyQ diseases may contribute for a deeper knowledge on the remaining neurodegenerative diseases, concerning both mechanisms and therapeutics.

In this review, we will outline recent efforts in drug discovery and development for polyQ diseases, including both types of strategies. We will start with a summary of preclinical studies and then move to the clinical trials performed to date in this group of disorders.

## A. Candidate-Based Approaches

### 1. Gene Silencing Through RNA Interference and microRNAs

The most straightforward therapeutic approach for polyQ diseases may be to decrease the expression levels of the mutant protein, and consequently prevent all the downstream deleterious events. Downregulation of the expression of the abnormal gene(s) was shown to be an effective strategy to halt disease progression in conditional tetracycline-regulated mouse and rat models of HD,<sup>86,87</sup> MJD/SCA3<sup>88</sup> and in a doxycycline-regulated mouse model of SCA1.<sup>89</sup> Upon shutting down mutant polyQ protein expression in these models, a reduction in the amount of NIIs as well as behavioral phenotype improvement was consistently observed. Another example was shown in a pan-neuronal conditional *Drosophila* model of SCA7 in which the expression of truncated protein was abolished leading to an increase in lifespan.<sup>90</sup> Once this proof of concept was obtained, several techniques targeting polyQ expression started being explored, including the use of small interference RNA (siRNA) and short-hairpin RNA (shRNA) molecules. The first description of interference RNA (RNAi) approaches for polyQ diseases was made in a cell-based model, in which the inhibition of expression of a truncated version of the human androgen receptor (AR) through double-stranded RNA strikingly reduced polyQ-mediated cell death, caspase-3 activity, and protein aggregation.<sup>91</sup> The applicability of RNAi *in vitro* was further described in an SCA1 mouse model using shRNA for human ataxin-1 (ATXN1), in which it was possible to observe an improvement in motor coordination, reduction in NIIs, and restored cerebellar morphology.<sup>92</sup> Later, microRNAs (miRNAs) suppressing the expression of ATXN1-encoding mRNA, and subsequently reducing the protein levels, were identified and shown to be efficacious in human cells.<sup>93</sup> Recently, the miRNA miR-25 was shown to be able to reduce both wild-type (wt) and polyQ-expanded mutant ataxin-3 (ATXN3) protein levels, therefore increasing cell viability, decreasing early apoptosis, and diminishing the accumulation of mutant ATXN3 protein aggregates in MJD/SCA3 cells.<sup>94</sup> In another miR-based approach, artificial miRNAs (non-allele-specific) targeting ataxin-7 (ATXN7) were injected into the retina eye or into the brain at the deep cerebellar nuclei (DCN) of a transgenic model of SCA7. The delivery of the miRNAs to the brain was shown to improve the motor phenotype and neuropathology, namely of cerebellar Purkinje cell dendrites. Furthermore, the artificial miRNAs injected into the retina of these mice were able to suppress  $\geq 50\%$  of ATXN7 levels, with no adverse effects in retinal functioning.<sup>95</sup> Extensive evidence supporting the utility of RNAi therapy was also provided in models of HD using different viral vector

to deliver shRNA silencing the mutant huntingtin (HTT). These treatments showed positive effects, improving the motor behavior performance and limb clasping, as well as reducing the NIIs in transgenic HD mice.<sup>96-98</sup> In the case of MJD/SCA3, using lentivirus-mediated allele-specific silencing of the mutant ATXN3 Nóbrega et al. were able to prevent the development of the motor and neuropathological phenotypic characteristics of a model of the disease.<sup>99</sup> However, the acute model system used did not mirror many aspects of the human disease, such as ubiquitous expression of the mutant protein, that in this case was restricted to the cerebellum, and the hyperactivity phenotype that is not seen in humans. Furthermore, the efficacy of the treatment was only assessed in a short period. To overcome some limitations of the model used, the same authors have used RNAi approaches in a transgenic mouse model of MJD/SCA3. In this study, the allele-specific silencing of mutant ATXN3 improved motor symptoms and ameliorated the neuropathological features related with MJD/SCA3.<sup>100</sup> In another study, using miRNAs and a different MJD/SCA3 mouse model,<sup>101</sup> with expression of the mutant ATXN3 under regulation of the endogenous promotor, a reduction of mutant ATXN3 was observed in the cerebellum of the animals injected with miRATXN3 as compared to controls, and no toxicity was found in the brain,<sup>102</sup> which led the authors to pursue with a chronic preclinical trial. In this preclinical trial, despite safety and lifelong suppression of ATXN3 expression in the cerebellum, the motor impairment was not ameliorated in treated MJD/SCA3 mice during the study (11 months of assessment) and the lifespan was not prolonged by treatment.<sup>103</sup>

In summary, therapeutic application of RNAi to polyQ diseases has progressed remarkably and reveals to be very promising due to the efficient and specific gene silencing. However, in order for this technology to be used to full advantage in the human patients, several key challenges still need to be overcome, namely regarding safety and delivery to the whole nervous system. For instance, oral administration is not possible with current technologies and RNAi formulations are limited to a few cell types and tissues. Also, tissue penetration, cellular uptake, and entrance into the cytoplasm, as well as off-target and long-term secondary effects, still need to be elucidated.<sup>104</sup>

## 2. Antisense oligonucleotide (ASO) Based Strategies

Another promising therapeutic approach to polyQ diseases, which are monogenic disorders, is targeting the causative gene directly, ideally without affecting the normal allele (allele-selective targeting) using oligonucleotides-based tools—ASOs-mediated strategy. Recently, Evers et al. proposed an innovative strategy for MJD/SCA3 treatment, which consists of mutant ATXN3 protein modification without affecting its normal function. This approach includes the removal of the toxic polyQ tract of ATXN3 using ASOs-mediated exon skipping, while keeping global levels of ATXN3 unchanged. In this study, the authors used an ASO that effectively removed simultaneously exons 9 and 10 of the ATXN3 mRNA(s) (the CAG repeat expansion is located at exon 10) in human fibroblasts. The removal of these exons did not have a negative impact in the ubiquitin binding capacity of ATXN3. To test the efficacy and safety of these ASOs *in vivo*, the authors proceeded to a single intraventricular injection of the ASOs in the brain of wt mice. After 7 days, the skipping efficacy was measured in the cerebellum of the mice, which demonstrated a reduction in the levels of exons 9 and 10 and not of the adjacent exons, suggesting the specificity of the ASOs used.<sup>105</sup> No data are available yet concerning the long-term safety or the efficacy of this approach *in vivo* regarding rescue of motor impairments and other neurologic symptoms.

In HD extensive research has also been performed using ASO approaches. In R6/2 mice, ASOs prevented the progressive brain atrophy.<sup>106</sup> Furthermore, ASO-mediated suppression of mutant HTT in the YAC128 mouse model was shown to improve the behavior phenotype and improve some changes in gene expression observed in the striatum.<sup>107</sup>

Interestingly, in the BACHD mouse model of HD, mutant HTT was suppressed for a long time, even after the termination of the treatment.<sup>106</sup> This study is in agreement with clinical data in ALS patients, where an ASO (known to reduce the mRNA and protein levels of mutant SOD1) was detectable in the spinal cord and in the brain of patients 3 months after an intrathecal injection of ASO.<sup>108</sup> The long half-lives of ASOs are particularly important in the case of polyQ diseases, given their slow progression, which require chronic treatment. Recently, a phase I clinical trial (NCT02519036) for HD, using huntingtin-targeting ASOs, has been initiated in early-stage HD patients, to assess safety and tolerability.

### 3. Preventing Mutant Protein Aggregation

Although the question of relevant aggregation species (discussed below) is continuously debated, it is more widely acknowledged that the polyQ proteins are toxic due to their aggregation propensity when compared to their wt counterparts,<sup>36,109</sup> leading to aberrant protein–protein interactions. Supporting the toxic gain of function hypothesis are the findings that HTT aggregates are toxic and are detected in the brain prior to neuropathological changes. Furthermore, the generation of a HD model expressing mutant HTT and lacking endogenous HTT showed that these animals presented the same HD-like characteristics when compared to animals expressing mutant HTT with a background of normal levels of endogenous HTT.<sup>110</sup> On the other hand, the loss of the protein normal function (through recruitment to inclusions or by abnormal conformation/interactions) might also contribute to the disease progression. Indeed, animal models carrying mutant HTT and those lacking HTT normal function show some similarities at pathological and behavioral levels, suggesting a contribution of a reduction in the normal function of HTT to the HD phenotype.<sup>111</sup> In accordance, a study in MJD/SCA3 showed that the lack of ATXN3 produced molecular alterations in a neuronal cell model that overlapped those seen in cells overexpressing expanded ATXN3, and also in neurons from a mouse model of MJD/SCA3, suggesting a contribution of an impaired function of ATXN3 to MJD/SCA3 pathogenesis.<sup>112</sup> In SCA1, in which the toxic gain of function hypothesis is well accepted, additional data suggest that perturbation of a normal ATXN1 function contributes to pathogenesis, namely through changes in specific interactions.<sup>113</sup> In this line, it was shown that lack of wt/endogenous copy of ATXN1, in a knock-in mouse model of SCA1 worsened the mouse phenotype and led to gene expression alterations.<sup>113,114</sup> Considering the arguments favoring the toxic gain of function and the loss of normal function of polyQ proteins, it seems reasonable to postulate that both may contribute to the disease progression, loss of function likely resulting from aggregation and impaired protein–protein interactions.

In normal conditions, cells possess a robust multilevel protein quality control machinery, to regulate homeostasis and guarantee that misfolded proteins do not accumulate and form insoluble aggregates.<sup>115,116</sup> This being a pathological hallmark of polyQ diseases, aggregation reduction was one of the first therapeutic approaches used in the polyQ field. Chaperones or heat-shock proteins (Hsp's) are crucial proteins that maintain the normal folding and/or the assembly of other macromolecular structures, and a defect in their normal function may per se have pathogenic implications.<sup>117–119</sup> A growing number of studies along the years reinforced the idea that overexpression of molecular chaperones in cellular and animal models of polyQ diseases has a protective effect, decreasing polyQ aggregation and/or neurodegeneration. In 1999, Warrick et al. showed that overexpression of Hsp70 was able to suppress polyQ toxicity in a *Drosophila* model even with no detectable effect on aggregation.<sup>120</sup> In cell models of SBMA, the overexpression of Hsp70 and Hsp40 suppressed aggregate formation and apoptosis<sup>121</sup> and enhanced the degradation of expanded AR.<sup>122</sup> The protective role of Hsp70 overexpression was also observed in SCA1 cell, fly, and mouse models.<sup>123,124</sup> In the case of MJD/SCA3, the overexpression of two Hsp40s, DNAJ-1 and DNAJ-2, was able to suppress the aggregation



of mutant ATXN3.<sup>125</sup> Another molecule with the ability to increase Hsp70, the co-chaperone Hip, was recently described to increase degradation of the mutant AR in cell culture and to rescue toxicity in an SBMA *Drosophila* model.<sup>126</sup> The heat-shock response (HSR; including the production of chaperones) can be induced through activation of heat-shock transcription factors (HSF), such as the heat-shock factor 1 (HSF-1), which has also been described to reduce aggregation and cell death in polyQ disease cell models.<sup>127–129</sup>

Beyond overexpression or constitutive activation of HSFs using genetic tools, increased HSR and HSP expression and protein folding can also be induced by a number of promising small chemical compounds. Trehalose, thought to act as a chemical chaperone, is able to stabilize polyQ proteins in their native conformation<sup>130,131</sup>; however, it is also known to be an autophagy inducer (discussed below).<sup>132</sup> This compound was shown to be efficient in HD cellular and animal models<sup>133</sup> and recently in SCA17 mice.<sup>134</sup> Geranylgeranylacetone, a molecule capable of enhancing the expression of Hsp70, Hsp90, and Hsp105 through induction of HSF-1 activity, proved to inhibit nuclear accumulation of mutant AR, to ameliorate motor behavior and increase survival in a SBMA mouse model.<sup>135</sup> Extensive evidence supports the use of two analog compounds, 17-AAG and 17-DMAG, also capable of upregulating Hsp40, Hsp70, and Hsp90, dependently on HSF-1 induction. They have been described to have promising effects on aggregation load, toxicity, and motor behavior in several cell, *D. melanogaster*, *Caenorhabditis elegans*, and mouse models of polyQ diseases.<sup>129,135–143</sup> Recently, the compound arimoclolmol has been shown to ameliorate the symptoms in SBMA and ALS mouse models, by boosting expression of chaperones Hsp70 and Hsp90, helping newly synthesized proteins properly fold.<sup>144,145</sup> Congo red, minocycline, and chlorpromazine are valuable aggregation inhibitors *in vitro* and were tested in mouse models of HD; however, mild or no improvement in phenotype was observed.<sup>146–148</sup> Cystamine may also reduce polyQ aggregation and increase survival by transglutaminase (TG) inhibition; TG has been proposed to crosslink expanded polyQ proteins and facilitate their aggregation<sup>149</sup> and was recently also linked to autophagy induction<sup>150,151</sup> (see below). This compound was beneficial for survival in HD mice by decreasing HTT aggregation,<sup>152</sup> but has not been tested in other polyQ diseases to date.

Another strategy in polyQ aggregation inhibition is centered on the use of small antibody fragments called intrabodies, which are a biotechnological tool that can be used to disturb, modulate, or define the functions, but also protein–protein interactions (and hence aggregation) of a wide range of target antigens at the posttranslational level.<sup>153</sup> In the case of HTT, intrabodies were described to reduce HTT aggregation and ameliorate neurological phenotypes in HD mouse models.<sup>154,155</sup> In MJD/SCA3, only one aggregate-exacerbating intrabody against fibrillar polyQ proteins was tested, causing an increased cytotoxicity and cell death in cells expressing mutant ATXN3.<sup>156</sup> Targeting the intermediate species in the process of aggregation and prevention of  $\beta$ -sheet dominant toxic conformation are also possible strategies that are discussed in the Section 2.B.1.

#### 4. Inhibiting the Cleavage of PolyQ Proteins

Expanded polyQ proteins are known to undergo proteolytic cleavage, generating smaller fragments that can have a higher toxicity<sup>157,158</sup> and/or more quickly aggregate.<sup>159</sup> The “toxic fragment hypothesis” suggests that proteolytic formation of polyQ fragments is a prerequisite of polyQ disease development. Therefore, modulation of the cleavage proteases’ activity or decreasing the levels of toxic fragments seems to be a reasonable therapy approach for polyQ diseases. Several proteolytic enzymes have been associated with the cleavage of polyQ proteins. The first described were the caspases in HD,<sup>160</sup> followed by calpains (a class of calcium-dependent cysteine proteases); however, even if these proteases contribute to the majority of cleavage effects, several other fragmentation events are observed which cannot be explained

by their proteolytic activity. Interestingly, a number of studies in different cell and mouse models demonstrated promising effects of caspase-1, caspase-3, and caspase-6 and calpain inhibition, as well as inhibition of matrix metalloproteinase (MMP) activity, in HD-associated toxicity.<sup>161–166</sup> The cleavage of mutant HTT by caspase-6 has been suggested to be required for the HD-related behavioral phenotype and selective neuropathology in the YAC128 mouse model of HD. Supporting this idea, caspase-6-resistant mutant HTT mice presented a delay in nuclear translocation of expanded HTT.<sup>167</sup> More recently, a transgenic mouse expressing the N-terminal 586 aminoacids of HTT, a putative cleavage fragment resulting of caspase-6 activity, showed behavioral deficits as well as nuclear accumulation of HTT aggregates. Although their phenotype was less severe than that of HD mouse models expressing shorter fragments, these studies suggest that the caspase-6 fragment may be an intermediate of the cascade leading to disease progression.<sup>168</sup> In DRPLA, atrophin 1 is also cleaved by caspases at aspartate 109, and cleavage site mutations reduce nuclear localization, aggregate formation, and cytotoxicity.<sup>158</sup> This was also shown in the context of MJD/SCA3, in which general caspase inhibition in a *Drosophila* model delayed the neurodegeneration progression caused by the expanded ATXN3 expression.<sup>169</sup> Targeting the expression of calpains was also proposed as a therapeutic approach in MJD/SCA3,<sup>170–172</sup> this inhibition being able to prevent the nuclear localization, aggregation, and toxicity of ATXN3. Recently, it was also described that knockdown of calpain (form *CalpA*) in a *Drosophila* model of HD leads to autophagy-dependent protection against polyQ toxicity.<sup>173</sup>

Caspase or calpain by small molecules inhibition could therefore have beneficial effects, through a decrease in generation of mutant proteins fragments. In fact, a general caspase inhibitor, z-Val-Ala-Asp-fluoromethylketone, extended lifespan and ameliorated the rotarod performance in R6/2 mice.<sup>166</sup> The combined administration of caspase-1 and caspase-3 inhibitors, Tyr-Val-Ala-Asp-chloromethylketone and Asp-Glu-Val-Aspaldehyde-fluoromethylketone, respectively, also increased the lifespan in HD mice.<sup>174</sup> z-DEVD-fmk, another caspase inhibitor, was found to inhibit HTT cleavage and toxicity in a cell model of HD.<sup>162</sup> When administered to a lentiviral mouse model of MJD/SCA3, BDA-410, a calpain inhibitor, was able to decrease the formation of both cleaved and full-length ATXN3 forms, as well as to reduce ATXN3 aggregation. Moreover, BDA-410 prevented motor deficits and preserved cerebellar morphology.<sup>175</sup> Finally, cystamine, a transglutaminase inhibitor (see above) able to increase lifespan of a HD mouse model, was also shown to reduce caspase activity.<sup>176</sup>

Another strategy is to modulate protease activity through alternative pathways. For example, the levels of caspase-1 and caspase-3 were decreased by treating R6/2 mice with the tetracycline derivative minocycline, retarding the disease progression and death.<sup>177</sup> Curiously, minocycline is a multifunctional compound, and was also described as an aggregation (see Section 2.A.2) and apoptosis inhibitor (see Section below 2.A.7). Recently a gene therapy approach to cleavage reduction was also proposed, using ASO-mediated mRNA (an approach discussed previously) and protein modification. The authors showed that modulating cleavage by inducing the skipping of exon 12 in HTT pre-mRNA inhibits the formation of an N-terminal fragment involved in HD toxicity.<sup>178</sup>

The generation and development of protease inhibitors able to prevent the conversion of polyQ proteins into more toxic fragments seems to be a promising therapeutic strategy. However, caution is warranted with these inhibitors concerning specificity, since there are several other essential signaling pathways in which proteases are involved<sup>179,180</sup> could also be affected. Ultimately, *in vivo* validation of the efficacy, safety, and tolerability of these approaches is required.

##### 5. Inducing Mutant Protein Degradation: UPS and Autophagy

Increasing the clearance of the mutant polyQ proteins to avoid their toxic downstream effects has been a focus of therapeutic strategies for several years. Cellular mechanisms for degrading

unwanted proteins include the UPS and autophagy. In several cellular and animal models of polyQ diseases, as well as in the patients' postmortem brains, polyQ inclusions co-localize with Ub and proteasome subunits, raising the possibility of UPS involvement in these diseases.<sup>123,181</sup> Some studies show that stimulating ubiquitylation and degradation of polyQ proteins through the UPS can rescue pathology in cell and animal models. By increasing UPS activity, Y-27632 (already known to be a rho-associated kinase inhibitor but also used in clinical trials as an anti-ischemic, anti-vasospastic, and anti-hypertensive agent) decreased polyQ toxicity in an HD *Drosophila* model<sup>182</sup> (an effect first identified in an unbiased drug screening, see Section 2.B.1), and reduced aggregation levels of mutant HTT and ATXN3 cell models, through its capability of modulating both UPS and autophagy.<sup>183,184</sup> Another compound shown to increase the enzymatic activity of the UPS was amilorid and its derivative, benzamil, also widely used in clinics as diuretics to treat or prevent hypokalemia in people with high blood pressure<sup>185</sup>; treatment with these compounds led to a reduction of mutant protein aggregation and toxicity in HD models.<sup>186</sup>

An additional strategy is the direct modulation of proteasome function. USP14 is a deubiquitylating enzyme (DUB) connected with the proteasome, which blocks the degradation of ubiquitylated substrates. IU1, a selective small-molecule inhibitor of USP14, was described to accelerate proteasomal degradation of polyQ proteins, such as ATXN3, but also of other proteins associated with neurodegenerative diseases such as tau, TDP43, and oxidized proteins.<sup>187</sup> However, since loss of function of *Usp14* in the mouse gives rise to cerebellar ataxia, some caution must be taken with this approach.<sup>188</sup>

E3 Ub ligases are enzymes that determine the substrate UPS specificity within the UPS and in ubiquitin signaling in general. The overexpression of Ub ligase CHIP (C terminus of Hsc-70-interacting protein) confers neuroprotection by enhancing ubiquitylation and consequent clearance of polyQ-expanded proteins, delaying the disease phenotype in SCA1, MJD/SCA3, and SBMA animal models.<sup>189–191</sup> Parkin, another E3, was also shown to ubiquitylate ATXN3 reducing polyQ toxicity in cells.<sup>192,193</sup> More recently, it was shown that the Nrf1 and Nrf2 transcription factors (known to regulate the oxidative stress response)<sup>194</sup> can also regulate the UPS.

Sulforaphane is a small molecule capable to induce Nrf2 activation, and therefore increase proteasome levels and activity<sup>195,196</sup> as well as enhance UPS function *in vivo*.<sup>197</sup> Through this action, this molecule was able to decrease the mutant HTT and alleviate polyQ toxicity in neuronal mouse cells.<sup>197</sup> These studies suggest that the UPS is a versatile system that can be targeted to increase the clearance of polyQ-expanded proteins, the main challenge being modulating this system in an effective and specific manner.

Autophagy is another prominent therapeutic target for the degradation of aggregated proteins, and it is negatively regulated by the mammalian target of rapamycin (mTOR).<sup>198</sup> Upregulation of autophagy by inhibiting mTOR with rapamycin showed beneficial effects in different animal models of neurodegenerative diseases by enhancing autophagy function.<sup>199–203</sup> For example, a rescue of polyQ toxicity in cell, fly, and mouse models was observed upon rapamycin treatment, in parallel with an increase in the clearance of both soluble and insoluble species of the expanded polyQ proteins.<sup>204,205</sup> Curiously, a selective ATP-competitive small molecule known as torin1 has been described to inhibit mTORC1 activity and consequently increase autophagy to a higher extent than rapamycin<sup>206</sup>; however, it still needs to be tested in polyQ disease models. Also tackling mTOR inhibition, Ravikumar et al. showed that by increasing intracellular glucose levels, mutant HTT clearance was enhanced and mutant HTT aggregates decreased.<sup>205</sup>

Autophagy can also be modulated independently of mTOR by the use of several compounds, such as lithium chloride (LiCl), sodium valproate, carbamazepine, rilmenidine, and other molecules that inhibit inositol monophosphatase.<sup>207,208</sup> Since some of these compounds

have been used for several years to treat human CNS and heart diseases, they may be easily translated to the clinics if they have promising effects in polyQ diseases. LiCl treatment ameliorated motor performance and depression in an HD mouse model upon postsymptomatic treatment, with no effect in survival.<sup>209</sup> In SCA1, treatment of the knock-in mouse model with LiCl also improved the neurological and pathological phenotypes.<sup>210</sup> In contrast, in the case of MJD/SCA3, LiCl therapy led to no overall improvement in motor function in the CMVMJD135 mouse model<sup>211</sup> nor in patients (see Section 3). Rilmenidine is an antihypertensive agent that was also tested in an HD mouse model, leading to amelioration of the disease symptoms and also to a reduction in the mutant HTT levels.<sup>212</sup> Other approaches may consist of modulating the expression of specific proteins or molecules known to induce autophagic degradation.<sup>213,214</sup> For instance, upon *C.elegans bec-1*, *Ce-atg7*, and *Ce-atg18* gene knockdown or knockout, the polyQ aggregate formation and polyQ-expanded protein toxicity increased.<sup>215</sup> Therefore, modulating these autophagic genes in the opposite direction could enhance the autophagic polyQ degradation. Overexpression of histone deacetylase 6 (HDAC6) led to an induction of the autophagy process compensating for UPS impairment in an SBMA *Drosophila* model.<sup>216</sup> More recently, the lentiviral vector-mediated overexpression of beclin-1 was described to enhance autophagy and to be protective in neuronal and in lentiviral-based rat model of MJD/SCA3.<sup>217</sup> Interestingly, 17-DMAG, an HSP90 inhibitor predicted to induce chaperone expression and thus promote mutant protein refolding and/or UPS degradation, was shown to function as an autophagy inducer in the CMVMJD135 mouse model of MJD/SCA3, improving motor uncoordination, and reducing NIIs.<sup>142</sup>

Trehalose, a compound already described in the above section as a chemical chaperone able to influence protein folding, is also an mTOR-independent autophagy inducer that can enhance clearance of aggregation-prone mutant HTT<sup>132</sup> and ATXN3<sup>218</sup> and protect against apoptotic insults in cells via its autophagy-inducing properties.<sup>132</sup> The combinatory effects of its autophagy-inducing and chemical chaperone activities, together with its lack of toxicity, places trehalose as a potentially interesting compound for polyQ disease therapy.

A combination treatment strategy for inducing autophagy to a higher level may also be considered. The combination of lithium or L-690,330 with rapamycin was shown to lead to enhanced clearance of mutant HTT and higher protective effects in HD cell and fly models, compared to treatment with either drug alone.<sup>207,219</sup> The lithium and rapamycin combination treatment was also a proposed approach (considering the extra protective effects of GSK-3 $\beta$  inhibition by lithium) that appeared to be cytoprotective in cell models of HD.<sup>219–221</sup> Studies in an MJD/SCA3 mouse model, however, did not confirm the efficacy or safety of a similar combination of lithium with CCI-79, a rapamycin analog, which led to neurotoxicity.<sup>222</sup>

Additional relevant studies on autophagy were also reported using unbiased drug screenings as discussed below. Overall, the UPS and autophagy induction strategies seem to be attractive; however, it is important to identify new safer and more tolerable small-molecule autophagy modulators to be used in the chronic treatment of poly Q diseases.<sup>223</sup>

## 6. Mitochondria Stabilization and Reduction of Oxidative Stress

Mitochondrial dysfunction and oxidative stress play an important role in polyQ pathogenesis. Several compounds improving energy metabolism defects or reducing oxidative stress have been successfully tested in cellular and mouse models of polyQ diseases (discussed below). Although the degree of oxidative stress involvement is not completely known, and while this can be seen as a very “downstream” event in the pathogenesis of polyQ diseases, antioxidant treatment may be able to exert important neuroprotective effects. Coenzyme Q10 (CoQ10) is a cofactor of the electron transport chain that supports mitochondrial energy generation, preserves cell viability, acts directly as a potent antioxidant *in vivo*, and induces increase of mitochondrial vitamin E

concentrations.<sup>224</sup> Cotreatment with CoQ10 and remacemide, an NMDA receptor antagonist, improved neurological symptoms and extended survival in different models of HD.<sup>244</sup> In contrast, this combinatory approach showed no overall effect in HD patients<sup>225</sup> (see Table III). CoQ10 alone was also able to reduce weight loss and NIIs as well as greatly improve the motor performance in the R6/2 mouse model of HD.<sup>226,227</sup> Supplementation with CoQ10 together with creatine, which provides a source of energy to compensate for reduced efficacy of mitochondria, also revealed additive neuroprotective effects in HD models.<sup>228</sup> Creatine supplementation per se was also able to improve brain pathology, phenotype as well as increase survival in the R6/2 mouse.<sup>229</sup> Also in the N171-82Q model of HD, survival was extended with creatine treatment.<sup>230</sup> Despite the disappointing results in one human study that showed no effect of creatine in HD patients,<sup>231</sup> a more recent study showed positive results in HD presymptomatic carriers. In this study, 60 patients (including diagnosed premanifest and nontested at-risk) were given a high dose of creatine for 18 months that showed to be safe and well-tolerated by the patients. Interestingly, neuroimaging in these patients demonstrated treatment-related slowing of cortical and striatal atrophy at 6 and 18 months, suggesting creatine as a promising therapy for HD if initiated early on<sup>232</sup> (see also Table III).

Resveratrol, a natural phenol with antioxidant and sirtuin modulation activities, reduced mutant polyQ toxicity in neuronal cells and *C. elegans* model of HD.<sup>233,234</sup> Recently, Cunha-Santos et al. demonstrated that this antioxidant, also known as sirtuin 1 (SIRT1) activator, improved balance and motor deficits in a MJD/SCA3 mouse model, when administered after disease onset. This study pointed SIRT1 modulation by resveratrol as a potential therapeutic target for MJD/SCA3.<sup>235</sup>

Tauroursodeoxycholic acid (TUDCA) is a hydrophilic endogenous bile acid with multiple neuroprotective properties, including antioxidant and anti-apoptosis activities. This compound was shown to decrease the apoptosis of striatal neurons and to improve motor and cognitive deficits in HD mouse models.<sup>236</sup> Several other molecules with known antioxidant activity, such as melatonin, selenium, quinolinic acid, idebenone, lycopene, lipoic acid, pyruvate, and L-carnitine, also proved to exert neuroprotective effects in HD models (reviewed in 237).

Also related with this group of targets is the Nrf2/antioxidant response element (ARE) signaling pathway, which is involved in antioxidant and anti-inflammatory responses.<sup>194</sup> For example, the triterpenoids CDDO-ethyl amide and CDDO-trifluoroethyl amide were described to upregulate Nrf2/ARE genes, thereby reducing the oxidative stress and striatal atrophy, improving the motor deficits, and increasing lifespan in the N171-82Q mouse model of HD.<sup>238</sup> Together, these results indicate that a therapy based on antioxidants and other promising molecules capable of improving mitochondrial function and reducing oxidative stress could be effective for polyQ diseases, particularly for HD.

## 7. Transcription Modulation

The current conceptual model is that mutant polyQ proteins frequently interact abnormally with histone acetyltransferases (HATs) and/or histone deacetylases (HDACs), changing physiological histone modification patterns and thus altering gene expression.<sup>239</sup> The normalization of the dysregulated gene transcription, usually by targeting histone acetylation and methylation, has been a growing field of research. The inhibition of HDAC activity, as a repressor of transcription, has been proposed as a transcription normalization method, and is the most widely tested in mouse models of polyQ diseases. Although HDAC inhibitor (HDACi) molecules frequently lack specificity, because they may also perturb the expression of other unrelated genes, some of them do exhibit promising therapeutic properties.

Molecules with HDACi activity such as sodium butyrate (SB), phenylbutyrate, suberoylanilide hydroxamic acid (SAHA or vorinostat), and valproic acid (VPA) or its salt derivative—sodium valproate, which are proposed to increase gene expression (albeit in a very nonspecific manner), have shown efficacy in a variety of polyQ disease models. Pharmacological treatment of the R6/2 transgenic mouse model of HD with SB improved body weight loss and motor phenotype, significantly extended the lifespan by more than 20%, and delayed the neuropathological damage; this was proposed to happen through the increase in acetylation of H3 and H4 and also of the transcription factor SP1.<sup>240</sup> An improvement in neurological phenotypes together with an increased acetylation of histones was also observed in transgenic mouse models of SBMA,<sup>241</sup> DRPLA,<sup>242</sup> MJD/SCA3,<sup>243</sup> and in a conditional pan-neuronal *Drosophila* model of SCA7<sup>90</sup> treated with SB. In spite of significant improvements in many neuropathological phenotypes, SB was not able to reduce the mutant polyQ protein aggregation in some of the models,<sup>240,241</sup> apparently acting downstream or independently of the aggregation component of the disease. Another HDACi, phenylbutyrate, reduced brain atrophy and ventricular enlargement, and improved lifespan in the N171-82Q transgenic model of HD.<sup>244</sup> SAHA has been tested in the R6/2 model of HD showing beneficial effects in the motor phenotypes and neuronal atrophy, however with no improvement in body weight. Like SB, SAHA rescued the hypoacetylation of histones in these animals, but had no effect on polyQ aggregates.<sup>245</sup> More recently, the same authors demonstrated that SAHA decreased HDAC2 and HDAC4 at the protein level in the cortex and brainstem, but not at the mRNA level.<sup>246</sup> SAHA was also described to induce autophagy,<sup>247,248</sup> which could contribute to its beneficial effects in polyQ disease models; however, the lack of effect on aggregation suggests the opposite in this case. VPA (or its salt—sodium valproate with pharmacokinetic similarity) is a well-tolerated antiepileptic drug, also with HDACi properties. This compound (or its salt) has been proven to delay the disease onset, reduce neurological deficits, and/or prolong survival in several models of neurodegenerative diseases, including HD, SBMA, and ALS.<sup>249-251</sup> In MJD/SCA3, VPA (a multitarget compound) was reported to alleviate neurodegeneration in a *Drosophila* model of the disease<sup>252</sup> and attenuate mutant ATXN3-induced cell toxicity in a human neuronal cell model,<sup>253</sup> while only mild effects in the CMVMJD135 mouse model.<sup>254</sup> In contrast, safety and efficacy of VPA was suggested in a clinical trial with MJD/SCA3 patients (see Section 3).<sup>255</sup> Thomas et al. successfully treated the R6/2 model of HD with a benzamide-type HDACi, HDACi 4b. This compound showed a lower toxicity *in vitro* when compared with previously tested HDACis, together with prevention of motor deficits and neurodegenerative processes in a postsymptomatic treatment approach.<sup>256</sup>

Other chromatin remodeling approaches, though not so well studied as the previous ones, comprise for instance the repurposing of chromomycin and mithramycin antibiotics, which were able to rescue the downregulation of a subset of affected genes, improve motor and neuropathological phenotypes, and increase the lifespan of transgenic mouse models of HD.<sup>257,258</sup> Another compound shown to alleviate the transcriptional dysfunction of HD models was the phosphodiesterase type IV inhibitor rolipram, which increases the phosphorylation and activity of CREB. Rolipram ameliorated neuropathology, slowed progression of neurological phenotype, and extended lifespan in R6/2 mice. Additionally, BDNF levels, which are decreased in this HD mouse,<sup>259,260</sup> were increased in treated mice through restored CREB function.<sup>261</sup> A subsequent study showed that rolipram also prevented the sequestration of CREB-binding protein (CBP) into NIIs.<sup>262</sup> Dimethylcurcumin (ASC-J9), a drug that specifically disrupts the interaction of the mutant AR with CBP, was shown to be symptomatically beneficial in the AR-97Q SBMA mouse model.<sup>263</sup>

In spite of the promising results obtained with HDCAi's in the polyQ disease field, a major drawback of this strategy is that it still lacks specificity. In addition to their modulation of the transcriptional defects, HDCAi's can also increase the acetylation of other nonhistone

proteins, such as tubulin<sup>264</sup> and Hsp90,<sup>265</sup> and modify levels of HSPs<sup>266,267</sup> via transcription-independent mechanisms. While some of the effects can add to their beneficial action, other issues stickle the translation of the promising results to the clinical context, contributing to some associated toxicity. Further studies of their actual molecular mechanism of action, and development of more selective HDACi's with less toxicity, might offer better opportunities.<sup>268</sup> Also, and assuming the increasing knowledge on HATs and their function in neuronal survival, the stimulation of HAT function appears to be another new therapeutic possibility in this field.<sup>269</sup> Lastly, more detailed studies on the epigenetic mechanisms underlying polyQ diseases may be relevant for the development of future therapeutic approaches.

### 8. Other Therapeutic Targets

The overactivation of NMDA glutamate receptors and consequent excitotoxicity leading to neuronal death has been proposed to play a role in the pathogenesis of HD.<sup>270</sup> Supporting this concept, the use of compounds that block the excessive glutamate release, as riluzole, has shown efficacy in HD mouse models concerning survival, motor performance, and aggregation.<sup>271</sup> A small study in human HD patients (see Table III and Section 3) showed some benefic effects in chorea intensity.<sup>272</sup> Yet, a larger and longer study demonstrated no effect of riluzole in HD patients,<sup>273</sup> suggesting that riluzole treatment may not useful in these patients. Postsymptomatic treatment of a conditional mouse model of MJD/SCA3 with riluzole led to no improvement of the motor uncoordination; intriguingly, riluzole chronic administration decreased the Calbindin expression in Purkinje cells of the cerebellum, suggesting possible toxicity.<sup>274</sup> In contrast, a randomized clinical trial in SCA patients of different etiologies showed the short-term efficacy (4 and 8 weeks) of riluzole in reducing disease symptoms. This was confirmed in a 12-month, multicenter, double-blind randomized trial including 55 patients, also with different types of hereditary ataxia, including some with polyQ-related ataxias.<sup>223–225</sup> Since MJD/SCA3 patients were not included in the abovementioned SCA clinical trials, the results of preclinical and clinical trials are not really conflicting; however, they suggest that further studies may be required with genetically homogeneous patient populations, in which attention should also be paid to the interpretation of the SARA score outcomes, trial design, sample sizes, and patient followup, as was discussed by several authors<sup>223–225</sup> (see Section 3).

Furthermore, two other compounds affecting the glutamate levels in the synaptic cleft, LY379268 and 2-methyl-6-(phenylethynyl)-pyridine, significantly increased the lifespan of R6/2 mice.<sup>275</sup> Administration of remacemide (NMDA antagonist) and/or of CoQ10 also prolonged the lifespan of HD mice.<sup>226</sup>

Recently, administration of the  $\beta$ -lactam antibiotic ceftriaxone, known to promote synaptic glutamate clearance by astrocytes, thereby reducing  $\text{Ca}^{2+}$  influx, was shown to improve behavior deficits in SCA28 mice when administered either prior to or after symptom onset, showing an additional potential strategy for treatment.<sup>276</sup>

Another option is apoptosis inhibition; anti-apoptotic drugs, such as minocycline and caspase inhibitors have shown promise in polyQ animal models. Administration of minocycline to R6/2 mice extended their survival,<sup>174</sup> and when combined with CoQ10 further improved survival, neuropathology, and motor phenotype when compared with any of the treatments alone.<sup>277</sup> However, long-term studies will be required to find out possible side-effects associated with suppression of these key cellular pathways at the organism level.

Other still emergent strategies for polyQ diseases may comprise neural cell replacement therapy and neuroprotection strategies. The replacement of dead and/or damaged neurons by new neural cells with the proper ability to integrate, function,<sup>278</sup> and also contributing to neurogenesis and the production of trophic factors<sup>279,280</sup> may provide additional neuroprotection and promote the reestablishment of neuronal homeostasis. The attractiveness of these strategies

lies partially in the fact that they might be effective even after disease manifestation, but many technical hurdles are still in the way of their translation to the clinic.<sup>281–283</sup>

### ***B. Unbiased Drug Screenings (Hypothesis-Free Approaches)***

Candidate-based drug design and testing, although a theoretically rational approach, is only one of the possible strategies for drug discovery, and although this approach can offer promising results, it is limited to known targets and drugs and it cannot unravel novel targets and disease pathways.

In the last years, unbiased drug screenings for polyQ therapy discovery are gaining relevance due to their numerous advantages and the novel technical possibilities for screening. High-throughput screening (HTS) allows the unbiased testing of the effects of thousands of compounds in one or more biological assays. The small molecules used in HTS's are often readily commercially available and are generally classified into three groups: (i) Food and Drug Administration (FDA)/European Medicines Agency (EMA) approved compounds, which comprise bioactive, natural products and synthetic small organic molecules already in use in humans, being studied with the aim of repurposing or repositioning for different diseases than those they have been used on; (ii) novel synthetic compounds, comprising a vast collection of novel organic molecules; and (iii) novel natural products. The FDA/EMA-approved compounds have the potential to rapidly translate into the clinic and of a faster interpretation of pathophysiological mechanism due to previous knowledge on their targets and mechanisms of action. The synthetic compounds may comprise molecules for additional targets can be easily improved and modified to increase potency and reduce side effects, being produced at significant lower costs. The natural products may also contain novel structures and may be superior in terms of biological activities and chemical complexity but are usually associated with higher production costs and/or limited availability and extract reproducibility challenges. However, they are an excellent starting point for novel compound discovery.

Besides the compound library selection, the most critical step is the choice of the biological readout, which must be key to the disease process and be compatible with automation. In polyQ diseases, drug discovery is hampered by the lack of robustly validated targets and the incomplete knowledge of the molecular pathways. Nevertheless, the strategy consists of screening against a specific disease hallmark in the available cellular or simple/invertebrate animal models, which need to recapitulate at least some disease-associated characteristic. In these diseases, the most common target has been polyQ protein aggregation, which is an important aspect of cellular pathology, either as a primary event or as a robust indicator of abnormal protein conformation and of cellular and proteostasis impairment. Other described targets for polyQ HTS have been cell death, mutant protein clearance (degradation), transcriptional dysregulation, and other events leading to toxicity. Any such model will have intrinsic advantages as well as limitations. A summary of described HTS's published to date in polyQ diseases is presented in Table II. Although the majority of the HTS's have been centered on HD, several concepts could potentially be applied to the other polyQ diseases.

#### *1. Aggregation Assays*

The first drug screenings were planned to identify compounds that would avoid the formation of HD aggregates or disrupt them *in vitro*. Examples of these assays include aggregate quantification by filter retardation assay with fluorescent ELISA readouts.<sup>284,285,287,290</sup> Although optimization of this method required much more time than the current HTS's, Heiser et al. were able to screen 184,880 small molecules and identified 25 benzothiazoles able to inhibit aggregation of the HTTQ51 protein, some of them being already described to be promising for



**Table II.** Chemical HTS's for PolyQ Diseases

Phenotype	PolyQ construct	Assay system/readout	Compounds tested	Hits	Secondary assays	Ref.	
Aggregation	GST-HTT exon 1 Q51	<i>in vitro</i> screen by filter retardation assay	184,880 small molecules	Benzothiazoles	Immunoblotting, electron microscopy, mass spectrometry, 293 Tet-Off cells	284	
	GST-HTT171 Q58	<i>In vitro</i> screen by filter retardation assay	1040 FDA-approved drugs and bioactive compounds	Gossypol, gambogic acid, juglone, celestrol, sanguinarine and anthralin	Hdh-Q111/Q111 striatal cells	285	
	GST Q62	<i>In vitro</i> screen	Phage display library	Six tryptophan-rich peptides (polyQ-binding peptide 1)	PolyQ turbidity, CFP/YFP FRET in HD COS-7 cell model, cell death	286	
	AR127 Q65	<i>In vitro</i> screen by CFP/YFP FRET assay	2800 biologically active compounds	Y-27632	HD <i>Drosophila</i> model	182	
	GST-HTT exon 1 Q51	<i>In vitro</i> screen by filter retardation assay	11 small molecules	Congo red, thioflavine S, chrysamine G and direct fast yellow	Electron microscopy, SDS/PAGE, and MS and HD COS-7 cells	287	
	HTT <sup>NT</sup> fragment	<i>In vitro</i> screen	15 peptides	HTT <sup>NT</sup> -related inhibitor peptides	SH-SY5Y cells	288	
	HTT Q72-Luciferase	HEK-293 cell-based screen by luciferase activity and FRET	2687 small molecules	Leflunomide and teriflunomide	Size and number of aggregates upon treatment and cycloheximide (CHX)-chase experiments	289	
	Aggregation and toxicity	GST-HTT exon 1 Q51	<i>In vitro</i> screen by filter retardation assay	5000 natural substances	EGCG and related polyphenols	HD yeast and <i>Drosophila</i> models	290
		AR127 Q65	HEK-293 cell-based screen by CFP/YFP FRET	3 libraries (4140 compounds)	EGFR inhibitor #3, EGFR inhibitor #4, gefitinib, nadolol, fosfosal, piceatannol, levonordefrin, todralazine, hydralazine, molsidomine, Ac-YVAD-cmk	Aggregation in PC12 cells, phenotype in HTT exon 1 in <i>Drosophila</i>	291
		HTT Q103-EGFP	Yeast cell-based screen by growth and EGFP fluorescence	16,000 compound library	C2-8	Microscopy and PC12-Q103 cells, HD COS-7 cells, brain-slices and HD <i>Drosophila</i> and R6/2 models	292
ΔProQ103		Yeast-based assay by growth readout	11,000 natural extracts compounds	Actinomycin D	Chaperone levels and PC12 cell aggregation	293	

Continued

Table II. Continued

Phenotype	PolyQ construct	Assay system/readout	Compounds tested	Hits	Secondary assays	Ref.
Cell death	AR Q112	HEK-293 cell-based assay by caspase-3 activation and fluorescence	1400 compounds	Nerifolin, Peruvoside, Digitoxin, Suloctidil	Rescue of Q112-related cell death by FACS	294
	HTT exon 1 Q103-EGFP	PC12 cell-based by LDH release and absorption	1040 compounds	12 compounds including cannabinoids and caspase inhibitors	NA	295
	HTT Q148	Inducible PC12 cell-based by LDH release and absorption	1040 compounds	Nipecotic acid, Mycophenolic acid, activenin, isoproterenol HCl and Propafenone	HTT aggregation	296
	HTT Q120	Temperature sensitive ST14A striatal cell-based assay by calcein AM cell viability and fluorescence	43,685 compounds	29 compounds (R1-R29)	Survival of Q103-related cell death in PC12, yeast, <i>C. elegans</i> and <i>Drosophila</i> and brain slices	297
Cell death and toxicity	HTT exon 1 Q25 and Q103-EGFP	PC12 cell-based screen by cycloaddition chemistry and in-gel fluorescence scanning	68,887 synthetic and natural compounds	16F16, thiomuscimol, cystamine	HD ans AD cortico-striatal brain slice models	298
Toxicity	HTT480 Q68	Striatal neuronal primary culture-based screen	40,000 compounds	NA	NA	299
	HTT190 Q8 and Q73	Cortical and Striatal neuronal primary culture-based screen by fluorescence scanning	400 small molecules	Inhibitors of Rho kinase, phosphodiesterase, adenosine 2A receptor and IKK $\beta$	NA	300
	HTT190 Q73	Rat brain slice-based screen	74 drug-like compounds	Inhibitors of IKK complex, CXCR3 chemokine receptor, c-Jun	NA	301
	HTT 588 C38 and Q15	<i>Drosophila</i> primary neuronal culture-based screen	2600 small molecule	N-terminal kinase and adenosine 2A receptor Camptothecin, OH-camptothecin, 18 $\beta$ -glycyrrhetic acid and carbenoxolone	HD <i>Drosophila</i> model	302
Clearance (degradation)	HTT573 Q72	HN10 neuronal cell-based screen by time-resolved FRET	10,000 natural compounds	TSA analogue, staurosporine, anisomycin, cycloheximide, borrelidin, BAY 61-3606	NA	303
	HTT573 Q25 and Q72	HN10 neuronal cell-based screen by time-resolved FRET	2 $\times$ 10 <sup>6</sup> compounds	Heat shock protein 90 inhibitors as NVP-AUY922	HdhQ150 embryonic stem cells and embryonic stem cell-derived neurons	304

Continued

Table II. Continued

Phenotype	PolyQ construct	Assay system/readout	Compounds tested	Hits	Secondary assays	Ref.
	Q103-EGFP	Inducible PC12 cell-based by protein concentration with EGFP fluorescence	37,000 synthetic compounds	31 compounds	$\alpha$ -complementation, Q103-related cell death	305
	NA	Yeast-based assay by growth readout	50,729 compounds	21 SMIRs and 12 SMERS	PC12 (A53T $\alpha$ -synuclein) clearance, HD exon 1 (Q74)-EGFP, autophagy with embryonic fibroblasts, COS, HeLa cells and <i>Drosophila</i>	306
	293-ATXN3Q81Luc and 293-ATXN3Q261Luc	HEK293 cell-based assay as primary screen; levels of ATXN3/Luciferase fusion proteins are measured by chemiluminescence	2880 small molecules (800 of which are FDA-approved)	3 compounds (salinomycin sodium, AM251, aripiprazole). Only aripiprazole is FDA-approved	Organotypic mouse brain slice cultures as secondary screen; Western-blot, Immunofluorescence, TEM, Hits tested in YACMJD84.2 mouse and fly models of the disease	307
HSF-1 activation	NA	HeLa cell-based screen	$1 \times 10^6$ compounds	A1, A3, C1, D1, F1	HD mammalian cellular and <i>C. elegans</i> model	308
	NA	Yeast-based assay	10,000 compounds	HSF1A (TRIC/CCT complex)	HD mammalian cellular and <i>C. elegans</i> model	309
Transcription dysregulation	HTT Q138	Inducible HEK-293 cell-based assay by Luciferase reporter gene	24,000 compounds	47 compounds	Cell viability adenosine triphosphate assay in primary striatal neurons transfected with N171-Q82	310
Whole organism behaviour	HTT Q128	<i>Drosophila</i> neuron-based screen	521 quinazoline-derived compounds	EVP4593	YAC128 medium spiny neurons	311
	HTT exon 1 Q150	<i>C. elegans</i> ASH neuron-based screen by GFP expression	9 compounds	LiCl, TSA, SAHA and mithramycin	NA	312
	ATXN3 Q130	Neuronal dysfunction-based screen	1120 chemical and pharmacologic small molecules	11 validated hits, citalopram selected for mouse studies	<i>C. elegans</i> ATXN3 Q130 aggregation; motor impairment and ATXN3 aggregation in CMV/MJD135 mouse model	313

GST, glutathione-S-transferase; FDA, Food and Drug Administration; CFP/YFP, cyan fluorescent protein/yellow fluorescent protein; FRET, fluorescence resonance energy transfer; EGCG, epigallocatechin gallate; EGFR, epidermal growth factor receptor; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; LDH, lactate dehydrogenase; GFP, green fluorescent protein; LiCl, lithium chloride; TSA, trichostatin A; SAHA, suberoyl amide hydroxamic acid; NA, not available; SMER, small-molecule enhancer; SMIR, small-molecule inhibitor.

ALS treatment.<sup>314</sup> As a second assay, these compounds were tested in HEK293 cells expressing HTTQ51, the majority of them being toxic to cells. This emphasizes the risks of translation from *in vitro* to cell cultures and, ultimately, to the *in vivo* context. Another *in vitro* aggregation assay with a longer HTT fragment was performed by Wang et al., who identified 19 compounds inhibiting aggregation by more than 50%.<sup>285</sup> Curiously, one of the hits was celestrol, an anti-inflammatory and antioxidant compound that has also been proposed for AD therapeutics.<sup>315</sup> To incorporate cellular context, a pioneering screen to monitor polyQ aggregation in cells was developed using a fluorescence resonance energy transfer (FRET) based assay that is based on the transfer of energy between two fluorophores that are in close spatial proximity. With this method, Pollit et al. screened a 2800 compound library and identified the Rho kinase (ROCK) inhibitor Y-27632 as a hit compound<sup>182</sup> decreasing mutant AR aggregation; they subsequently validated its beneficial effects in a *Drosophila* model of HD. The discovery of the ROCK inhibitors in the context of aggregation allowed the characterization of the ROCK-profilin signaling pathway.<sup>316,317</sup> The FDA-approved ROCK inhibitor **HA-1077** was shown to rescue retinal degeneration in the R6/2 mouse model of HD, showing that ROCK signaling inhibition is an HD-relevant mechanistic pathway.<sup>318</sup> With the same strategy, the group screened a set of biologically active compounds and found hits capable of inhibiting the aggregation of a pure polyQ stretch in HEK293 cells, suggesting these compounds as essential tools for understanding and counteracting the aggregation mechanisms in polyQ diseases. Some of the compounds were also able to alleviate the neurodegeneration in a *Drosophila* model of HD.<sup>291</sup> Recently, Fuentalba et al. developed a robust and quantitative method to assess protein aggregation dynamics, using aggregation-sensitive luciferase-based reporter to quantify polyQ aggregation *in cellulo*, based on an expanded HTT fragment.<sup>289</sup> With this method the authors identified leflunomide and its active metabolite teriflunomide, as preventing incorporation of expanded polyQ proteins into aggregates. This compound could therefore hold promise for polyQ diseases, with the additional benefit that it was already tested in multiple sclerosis patients in a phase III clinical trial, resulting in the decrease of annualized relapse rate and the lesion volume; however its long-term safety profile remains unknown.<sup>319</sup> In addition, the efficacy of teriflunomide was also confirmed in yeast systems. This biological system is less susceptible to polyQ-mediated toxicity, and easily subject to genetic analysis, allowing the study of multiple cellular factors in the aggregation process. In 2005, a primary aggregation-based screen was performed with 16,000 compounds in a yeast strain, using a galactose-inducible HTT construct with 103Qs tagged with EGFP.<sup>292</sup> Nine hits were identified and microscopically validated. Four of the compounds were found to inhibit the aggregation of this fusion protein also in PC12 cells. However, the compounds' activity was weak in a cell-filter retardation assay, demonstrating that they did not interfere directly with polyQ aggregation. A recent study also used a yeast model of polyQ proteotoxicity to screen a large amount of natural product extracts, identifying actinomycin D as a strong inhibitor of polyQ aggregation, an effect that was accompanied by its impact in increasing the levels of different chaperones.<sup>293</sup> This compound also suppressed aggregation in mammalian cells, suggestive of a conserved mechanism and therefore placing this as an interesting compound for polyQ diseases. Efforts were also made to establish brain slice assays that could closely mirror the aggregation events in mouse models of HD,<sup>320-323</sup> and although the authors could confirm the anti-aggregation properties of Congo red in this system, the predictive value of this assay for the compounds' activity in animals and patients still needs to be confirmed.

Targeting intermediate species in the process of aggregation could also be a strategy for therapy. Taking this into consideration, the compounds able to decrease the oligomeric species rather than insoluble inclusions may be considered as more interesting therapeutic candidates. Indeed, a compound designated **B2**, was identified in a drug screening and shown to promote HTT aggregation and to decrease cytotoxicity in a cellular model of HD.<sup>324</sup> Following

studies also demonstrated the ability of B2 to increase aggregates in cellular models of SBMA, and significantly decreased the extent of alteration of the eye phenotype in a fly model of SBMA.<sup>325</sup>

Another example comes from the screening of a library of ~5000 natural substances, in which (–)-epigallocatechin-3-gallate (EGCG), a green tea polyphenol, was identified as an inhibitor of HTT aggregation *in vitro*. This compound reduced toxicity and aggregate load in a yeast model of HD and when administered in a *Drosophila* model of HD, it allowed a reduction in photoreceptor degeneration and motor impairment. EGCG seemed to exert its positive effect on aggregation *in vitro* by suppressing the formation of small oligomers and stimulating the formation of larger aggregation species.<sup>290</sup>

Additionally, some evidence supports the notion that the expanded polyQ segment confers a new three-dimensional conformation to the proteins.<sup>326</sup> The “Exposed  $\beta$ -sheet” theory is based on the possibility that expanded units of polyQ proteins establish strong hydrogen bonds with each other, leading to the formation of insoluble parallel  $\beta$ -sheet structures,<sup>327</sup> and ultimately to their accumulation as inclusion bodies in the neurons. Considering this hypothesis, compounds targeting the abnormal protein conformation and the subsequent  $\beta$ -sheet formation process might constitute a useful therapeutic strategy. Considering the existence of an antibody that recognizes the conformation of an expanded polyQ tract (1C2) in a specific manner, peptides that can specifically bind to pathogenic polyQ domains could inhibit the interaction of the monomers with each other and slow/prevent the aggregation and the neurodegenerative process.<sup>286,328</sup> Using a peptide library screening, Popiel et al. found the polyQ-binding protein 1 (QBP1) as an inhibitor of the toxic  $\beta$ -sheet conformational transition and aggregation of the expanded polyQ *in vitro*. The treatment of a *Drosophila* model of HD with this compound was shown to prevent conversion of the expanded polyQ into its aggregation-prone  $\beta$ -sheet conformation, resulting in a neuroprotective effect.<sup>328,329</sup> Nevertheless, this peptide was shown to be too large to cross the cell membrane and to reach the intracellular polyQ protein efficiently. The fusion of protein transduction domains (PTDs) enabled the intracellular delivery of QBP1, inhibited polyQ aggregation and suppressed polyQ inclusion body formation and cytotoxicity *in vitro*.<sup>330</sup> Oral administration of PTD-QBP1 suppressed polyQ-induced premature death and inclusion body formation in a *Drosophila* model of MJD/SCA3.<sup>330,331</sup> However, in the R6/2 mouse model of HD weight loss phenotype only improved when this molecule was administered via intraperitoneal injection, showing oral administration to be ineffective.<sup>332</sup>

Recently, another peptide tested as a candidate-based molecule, P42 (part of the endogenous HTT protein and lies within a region rich in proteolytic sites), was found to exert beneficial effects in a *Drosophila* HD model.<sup>333</sup> To further test the beneficial effects of P42, Arribat et al. tested it in the R6/2 mouse model of HD, using a systemic delivery route of administration. For that, they used a combinatory approach fusing P42 to the protein transduction domain of the HIV TAT protein, allowing the delivery to living cells via endocytosis. Also, they used a novel water-in-oil microemulsion drug delivery vector called Aonys, providing a transmucosal route of administration. P42 daily administration showed to be safe and to improve behavior deficits observed in the R6/2 mice as well as to reduce HTT aggregates and astrogliosis.<sup>334</sup>

Chen et al. generated chemically a library of 60,000 of peptoids (oligomers of N-substituted glycines) that was screened for molecules that specifically bound to an amino-terminal fragment of mutant HTT protein, in order to overcome delivery issues of the peptides. This screening resulted in the identification of HQP09. This peptoid prevented the aggregation of mutant HTT *in vitro* and in YAC128 medium spiny neuron (MSN) cultures MSN cultures, also stabilizing glutamate-induced  $\text{Ca}^{2+}$  signals and protecting them from glutamate-induced apoptosis. *In vivo*, intracerebroventricular delivery of HQP09 to the YAC128 HD model resulted in reduced accumulation of mutant HTT aggregates and improved motor performance.<sup>332</sup>

Diverse *in vitro* and cell-based assays have allowed the identification of small molecule aggregation inhibitors, some of which were also found to be active *in vivo* (e.g., in the *Drosophila* model). Despite the encouraging results with the aggregation assays, some limitations are still present. Particularly, the evaluation of the toxic effects of the compounds at early stages should be improved and the use of different model systems might contribute to variable outcomes of the same compound, suggesting that the same library of compounds should be tested in several model system of the disease in study.

## 2. Cell Death Assays

In addition to aggregation assays, more complex biological assays aiming at phenotypic endpoints associated with the disease process could be used. Although the exact role and timing of cell death in polyQ pathogenesis is still discussed,<sup>335,336</sup> this downstream consequence of toxicity is being used as an assay readout for the identification of novel small molecules. Caspase-3 activation is a key event in apoptotic cell death and its genetic or small molecule inhibition showed to be beneficial in cell culture and mouse models of HD.<sup>337,338</sup> A cell-based assay was used to identify inhibitors of caspase activation in a model of SBMA, in which expression of mutant AR Q112 induces caspase-3 activation and cell death.<sup>294</sup> A 1400-compound collection was screened and the researchers identified 15 compounds inhibiting caspase-3 activity by more than 70%; however, only four cardiac glycosides were confirmed to enhance cell viability and not cause cytotoxicity. Curiously, these compounds did not inhibit caspase-3 directly, suggesting that they target upstream players in the cascade of death pathway. Thus, the study of the mechanisms of action of these compounds could disclose new and unexpected biological targets in HD.

Apart from this cell death related molecular endpoint, there are also HTS's based on the phenotypic endpoint, cell death itself. Aiken et al. used an inducible PC12 cell model, with a previously described construct containing 103Q's,<sup>339</sup> which underwent rapid cell death, and quantified lactate dehydrogenase (LDH) release or measured mitochondrial activity with an MTS assay. With this model, the group screened 1040 compounds and identified 12 that completely rescued cell death. Among these compounds, cannabinoids ( $\Delta$ -8-THC,  $\Delta$ -9-THC, cannabidiol, and cannabidiol) were identified as efficient for protection against cell death,<sup>295</sup> through a possible mechanism related with their described antioxidant effects.<sup>340</sup> Another screen with the same set of compounds, in an inducible PC12 cell model, but with a construct with 148Qs, identified five compounds able to prevent cell death, three of them (acicvicin, nipecotic acid, and mycophenolic acid) also decreasing aggregation.<sup>296</sup> It is important to note that, although these two HTS's were performed with the same inducible model and the same compound library, no overlap was found in the hit compounds, with the exception of caspase inhibitors.

One cannot stress too much the importance of the cellular model and polyQ construct when designing the assay. Thus, it is valuable to perform HTS's in cell lines that mirror as much as possible the cell types known to be affected in the disease context. With that purpose, Varma et al. used a stable striatal neuronal cell line expressing N-terminal 548-aa fragment of HTT with 120Qs, which undergoes cell death upon serum deprivation at 39°C.<sup>297</sup> In a collection of 43,685 compounds, they identified 29 hits rescuing cell death specifically in mutant but not in parental striatal cells, suggesting that these compounds are specifically activated by mutant HTT and consequently are possibly relevant therapeutic targets. As secondary assay, the authors tested these 29 compounds in three additional HD models—PC12 cells, yeast, and *C. elegans*, four of them (R1, R2, R3, and R4) being active in all these models, and two of them also active in a HD rat brain slice assay. Although their mechanism of action is still under study, these compounds seem to specifically target mutant HTT toxicity across cell-, tissue-, and organism-based HD models.

### 3. Mutant Protein Clearance/Degradation Assays

A different strategy than those referred above is to perform an HTS directed at the cellular degradation of mutant polyQ proteins. With the purpose of identifying compounds promoting the clearance of the mutant polyQ protein in mammalian cells, Coufal et al. used the HTT gene with 103Qs in fusion with EGFP, placed under inducible control of an ecdysone control element in PC12 cells.<sup>305</sup> These authors screened a library of 37,000 compounds, basing their screen on fast degradation of the Q130-EGFP fusion proteins 24 hr after the inducer removal. Following toxicity studies, they identified 31 compounds that accelerated degradation of this protein, of which two were structurally similar compounds, A28 and A31. A second assay aiming to study these compounds in terms of their specificity for a mutant (Q79) or a wt (Q23) HTT protein in PC12 cells showed that A31 increased clearance and cell viability for the mutant HTT but not for wt protein.<sup>305</sup>

An HTS screening to search for modulators of autophagy was performed in a *Saccharomyces cerevisiae* model by Sarkar et al.<sup>306</sup> The mTOR kinase is a major negative regulator of autophagy,<sup>341</sup> and thus an interesting drug target in protein aggregation-related diseases, although the utility/safety of its chronic inhibition is under debate. In this HTS, 15,000 compounds were screened and the authors identified 12 novel small-molecule enhancers (SMERs) of autophagy, modulating the levels of HTT and A35T  $\alpha$ -synuclein, and 21 inhibitors of the cytostatic effects of rapamycin (SMIRs) in yeast. The SMERs were shown to be independent or functioning downstream of mTOR, and proved to attenuate mutant HTT toxicity in HD cell and *Drosophila* models.

Another cell-based assay through a time-resolved FRET analysis was performed by Paganetti et al., the main advantage of which was the possibility to adapt the design for protein visualization in intracellular imaging assays, with fixed and permeabilized cells.<sup>303</sup> To validate the system, the authors tested 10,000 compounds for their ability to modulate intracellular HTT levels, with an additional cytotoxicity readout. In this manner, by screening a natural product library, the authors identified six molecules (trichostatin A analogue, staurosporine, anisomycin, cycloheximide, borrelidin and BAY 61–3606) that efficiently reduced or increased the mutant HTT present in the cells.

Lately, an HTS using a hippocampal HN10 cell line expressing mutant HTT and time-resolved FRET assay, identified several hits, including Hsp90 inhibitors.<sup>304</sup> These compounds strikingly decreased mutant HTT levels without major toxic effects. The hit NVP-AUY922 was shown to enhance the mutant protein degradation also in HdhQ150 embryonic stem (ES) cells and ES cell-derived neurons. This compound significantly decreased the soluble full-length mutant HTT levels and enhanced mutant HTT clearance without induction of Hsp70, suggesting it is acting through a mechanism other than chaperone induction and making it a promising therapeutic molecule.

Recently, Costa et al. used a cell-based system for HTS and tested a library of compounds with the goal of reducing the levels of mutant ATXN3; after this primary screening, the authors performed a secondary screening using organotypic brain slice cultures from a transgenic mouse model of MJD/SCA3 (YACMJD84.2).<sup>101</sup> From 1,250 FDA-approved drugs, it was found that the atypical antipsychotic drug aripiprazole reduces the levels of mutant ATXN3 in both systems. This compound was further tested *in vivo* in the mouse and fly models of MJD/SCA3 increasing the longevity of the flies and reducing mutant ATXN3 species in the brain of the transgenic mouse model.<sup>307</sup>

### 4. Transcription Dysregulation Assays

As the knowledge of the pathogenic mechanisms of polyQ diseases evolves, more relevant and quantifiable readouts can be used for HTS's of compounds. Although the transcription

dysregulation of polyQ diseases is still not understood at the mechanistic level, this seems to be an important aspect of their pathogenesis. In this line, it is known that mutant HTT is able to sequester the CREB co-activator CBP through direct polyQ interactions leading to reduced CREB-mediated transcription.<sup>342</sup> Taking this into consideration, a recent study from Lazzeroni et al. described a novel phenotypic screening assay to identify modulators of huntingtin-induced transcriptional dysregulation.<sup>310</sup> The authors used a stable cell line expressing an inducible full-length mutant HTT gene (138Q), together with a reporter gene under the control of the cAMP responsive element (CRE), known to be perturbed by expanded polyQ. The full-length protein used in this study enabled a more disease-relevant cellular phenotype than that measured for models expressing exon-1 or other HTT fragments, previously described. As a proof-of-concept, upon treatment with Rho kinase inhibitor Y27632, one cellular clone showed reversible inhibition of HTT reporter activity and was then used for a HTS of 24,000 compounds; 64 of these compounds were identified to have different specificity levels for CRE-mediated transcriptional regulation, and secondary assays were performed to tackle their mechanisms of action and their effects on mutant HTT aggregation.

Recent studies have also been focusing on other parameters of polyQ pathogenesis and on candidate targets. One is the activation of the HSF-1, which has been shown to restore proteostasis in a wide range of neurodegenerative diseases.<sup>129,136,139,309</sup> A few small molecules were already identified as regulators of protein conformation through the activation of HSF-1 in HeLa cell<sup>308</sup> and yeast-based assays.<sup>309</sup> In spite of being a transcription factor-based assay, this study also has aggregation as a therapeutic target.

### 5. Whole Organism Based Screens

Although the majority of the HTS's are performed in a simple cell-based assays and the *in vivo* studies in rodent models are only used as a last part of the screening, before testing the compounds in clinical trials, some nonrodent *in vivo* models can actually be used for primary library screenings. Despite their higher cost and throughput limitations when compared to assays described above, *D. melanogaster* and *C. elegans* are two models that can be used for drug screenings, with the advantage of being multicellular organisms and displaying polyQ disease-related quantifiable phenotypes for HTS. PolyQ-associated phenotypes, as motor impairment, for instance, could be used as first line readouts in *Drosophila*<sup>311</sup> and *C. elegans* models<sup>141,313</sup> in the future of polyQ HTS's. One of the major concerns of *in vitro* and cell-based assays is whether the hits will be also effective (and not toxic) in a whole animal model. In this context, *C. elegans* is a powerful model for drug testing in whole animal context, due to its short life cycle, small size, and easy culturing in liquid medium. Several neurobiological and antiparasitic drug studies in *C. elegans* also provide a strong basis to use this organism in drug discovery identification.<sup>343,344</sup> Voisine et al. used a *C. elegans* model of polyQ neurotoxicity expressing N-terminal 171 aa fragment of human HTT with 151Qs in neurons, and mimicking the degeneration and cell death (ASH sensorial neurons) of the human disease, dependent on both age and the length of the polyQ tract.<sup>312</sup> The authors tested nine candidate compounds previously identified in cell cultures and animal studies in the *C. elegans* model and found that two FDA-approved compounds, LiCl and mithramycin, independently and in combination, alleviated HD neurotoxicity.<sup>312</sup> Due to its easiest genetic manipulation, *C. elegans* is also valuable to study the drugs' mechanisms of action by turning on/off possible targets, accelerating drug discovery for polyQ diseases. A neuronal dysfunction-based screen of FDA compounds in *C. elegans* model of MJD/SCA3 was recently described, in which 48 effective molecules were identified, including the selective serotonin re-uptake inhibitor—citalopram. This compound was highly effective in the rescue of locomotion impairment and also in the decrease of ATXN-3 aggregation. Following studies in a mouse model of MJD/SCA3 with citalopram also showed



an improvement in the locomotor impairment and a decrease in ATXN-3 aggregation, together with other neuroprotective effects.<sup>313</sup>

### ***C. Considerations on Drug Discovery Strategies in PolyQ Diseases***

In the last decade, a significant number of HTS assays have been established that lead to the identification of multiple of polyQ disease-modifying compounds, comprising both FDA-approved drugs and novel chemically synthesized or natural molecules. A further challenge is to test these molecules in clinical trials and use them as tools to enhance the understanding of the polyQ disease mechanisms, therefore unravelling novel therapeutical targets.

The hurdles for the novel molecules identified in large-scale screenings can be complex, as they need to pass several filters, including their analysis in different secondary assays in terms of toxicity, potency, metabolic stability, adequate pharmacokinetic profile, and, relevant to polyQ diseases, their capability to cross the blood–brain barrier (BBB).

In this perspective, the best candidates will be the FDA-approved compounds known to cross the BBB, which, once they prove to be effective in animal models, can be taken to clinical trials quite fast without lengthy safety studies—the re-purposing approach.<sup>345–348</sup>

A unifying handicap in polyQ disease therapeutics discovery is that mutant polyQ expression is associated with the disturbance of several cellular mechanisms. The multiplicity of affected pathways makes the process of drug discovery difficult since it may be hard to define the most important disease mechanism to target. However, the challenging but also productive pathway that has been travelled so far in this field, together with the fast technological evolution improvement in biotechnology, means that we will have the best tools to tackle the emerging and old questions, hopefully toward a significant advance in the development of therapeutic strategies for these devastating diseases.

Both approaches presented in this review (candidate-based and unbiased drug screenings) have their pros and cons in the discovery of new promising compounds for polyQ disorders. To date, it seems that more successful compounds were identified with candidate-based approaches since that was the primarily used strategy along the years. Yet, and despite being more expensive due to high technology requirements, unbiased drug screenings are gaining relevance, and already allowed the identification of promising compounds for which validation should be pursued in preclinical trials. These compounds may lead us to very relevant novel targets and may help to elucidate more precisely the mechanisms involved in the course of the disease.

However, a challenge still needs to be addressed for both strategies: results must be replicated in more than once in different models of a given disease. In this perspective, it is also intriguing that a large number of positive preclinical results published in the last years for polyQ diseases were not pursued and, more importantly, did not advance to Clinical Trials (see Section 3), which suggests a requirement for more rigorous studies with robust validation strategies but also more investment and effort in the establishment of drug discovery and development pipelines for these rare disorders.

### **3. OVERVIEW OF CLINICAL TRIALS IN POLYQ DISEASES**

Clinical trials are an essential step in the discovery of new treatments and provide the final evidence of a molecule's efficacy, contributing for the advancing of medical knowledge and patient care. Despite the enormous efforts in the past decades, there is no effective treatment for polyQ diseases. However, several clinical trials were performed and numerous are ongoing. In some of these, specific symptoms of these diseases could be attenuated (chorea, alertness, or palliative benefits).<sup>272,349–360</sup> The majority of the clinical trials published to date in polyQ

diseases are small studies with few patients, mostly open-label, observational and/or retrospective trials; hence, there is a clear need for novel well-powered randomized controlled trials (RCTs). These trials are more quantitative, comparative and controlled experiments in which the effect of a specific treatment may be determined with less bias. Randomization is also considered a powerful aspect of the experimental design reducing allocation bias and enabling the detection of the differences in outcome only attributable to the intervention. Table III summarizes the double-blinded, phase 2 and/or phase 3 randomized controlled clinical trials completed to date for polyQ diseases, with 20 or more patients, available at [clinicaltrials.gov](http://clinicaltrials.gov) and/or Pubmed (excluding open-label and retrospective trials). The main objective of this categorization was to cover the high-quality evidence RCTs.

Intriguingly, in the polyQ field very few of the clinical trials performed to date were based on *in vivo* preclinical data. Most compounds were chosen based on positive effects and safety of each specific drug in another disease or for other indication rather than on preclinical studies. A few of these, for instance, antihistamine, antidepressants, or cannabinoid agonists, did lead to partial improvements (see Table III). However, some examples of a progression from preclinical to clinical trials do exist. Watase et al. showed an improvement of neurological and pathological phenotypes in a knock-in mouse model of SCA1 when treated with lithium.<sup>210</sup> Thereafter, the same compound was tested in SCA1 (NCT00683943) and SCA2<sup>375,377</sup>; however, with only few or not relevant positive outcomes. Curiously, lithium carbonate was tested in MJD/SCA3 patients with only limited effects<sup>377</sup> on SARA scores and on disease progression; and only after this human clinical trial a study in a mouse model of MJD/SCA3 was published, with no positive results.<sup>211</sup> Another example was VPA, which was previously described to alleviate neurodegeneration in a *Drosophila* model of MJD/SCA3,<sup>252</sup> although with only mild phenotypic improvement in an MJD/SCA3 mouse model.<sup>254</sup> When administered to patients, VPA led to an improvement in locomotor function given by the decrease in global SARA score<sup>255</sup>; however, several methodological issues, namely, the very low number of patients in each group and the secondary effects observed, including dizziness and loss of appetite, should be taken into consideration in the interpretation of these results.<sup>255</sup>

In the case of HD, 10 out of 21 compounds tested were based on preclinical data, mainly in mouse and *C. elegans*. For example, fluoxetine, a common antidepressant, was shown to improve hippocampal-dependent cognitive and depressive-like behaviors in HD mice, as well as to rescue neurogenesis deficits and volume loss in the dentate gyrus of these mice.<sup>379</sup> In contrast, the clinical trials showed no overall effect of fluoxetine in total functional capacity (TFC) and in standardized neurological, cognitive, and behavioral ratings.<sup>365</sup> Duration and age at initiation of treatment might, however, have been a limiting factor in this (and other) trials. Another example is the case of creatine, which showed positive effects in several models of HD, both at the behavioral and molecular levels.<sup>228,229</sup> In spite of the controversy regarding clinical trials in HD patients, oral creatine administration was shown to increase the serum and brain levels of creatine as well as decrease the serum levels of 8OH2'dG, an indicator of DNA oxidative damage known to be markedly increased in HD patients.<sup>380</sup> Recently, an RCT in HD patients using creatine showed a slower cortical and striatal atrophy progression at 6 and 18 months of followup.<sup>232</sup>

For SBMA, one of two compounds studied in patients to date was tested in a mouse model of the disease. Leuprorelin showed promising results in an SBMA mouse model, reversing the behavioral and histopathological phenotypes.<sup>381</sup> However, when administered to patients, this compound did not show significant effects.<sup>373</sup>

Overall and despite many clinical trials performed, unfortunately none of the compounds tested to date proved to be the ideal candidate. These findings alert for the importance of gathering more preclinical data in the appropriate models in order to support the decision of a human clinical trial with candidate compounds. Issues also exist with rigor in preclinical

**Table III.** Completed RCTs for PolyQ diseases With Available Results at Clinicaltrials.gov, Clinicaltrialsregister.eu, and/or PubMed (1982–2016)

PolyQ disease	Therapeutic molecule	Mechanism of action	Treatment duration (weeks)	N	Overall efficacy	Ref.	Clinical trial code
<b>HD</b>	Latrepidine	Antihistamine, Mitochondrial membrane stabilizer	12.9	91	<b>Improvement in the MMSE scores mean</b>	349	NCT00497159
	Creatine*	Energy metabolism regulator	26	403	No significant effect in cognition or global function	361	NCT00497159
	Creatine*	Energy metabolism regulator	52	41	No significant effect in functional, neuromuscular, and cognitive status	231	NCT00592995
	Creatine*	Energy metabolism regulator	72	60	<b>Slow cortical and striatal atrophy at 6 and 18 months</b>	232	NCT00712426
	Pridopidine	Dopaminergic stabilizer	4	58	<b>Trend towards improvement in affective symptoms; no significant effect in voluntary motor symptoms</b>	350	2004-000394-60
	Nabilone	Cannabinoid CB1 and CB2 agonist	15	44	<b>Significant effect for total motor score, chorea, UHDRS cognition, UHDRS behavior and for NPI.</b>	351	NA
	Ethyl-eicosapentaenoic acid	Multiple targets Mitochondrial function	24	316	No significant effect in Total Motor Score 4 and in measures of function, cognition, or global impression	362	NCT00146211
	Atomoxetine*	Noradrenaline reuptake inhibitor	52	135	No significant effect on intention-to-treat (ITT) analysis	363	2005-002088-98
	Fluoxetine*	Selective Serotonin Reuptake Inhibitor	10	20	No significant effect in self-reported attention, executive neuropsychological, psychiatric and motor symptom scores	364	NCT00368849
	Citalopram*	Selective Serotonin Reuptake Inhibitor	16	30	No significant effect in total functional capacity (TFC) and in standardized neurological, cognitive, and behavioral ratings	365	NA
	Lamotrigine	Glutamate release inhibitor	20	33	No significant effect in executive functions	366	NCT00271596
	Riluzole	Glutamate release inhibitor	30	64	No significant effect in delaying disease progression assessed by TFC. <b>Symptomatic improvement in some patients and a trend towards chorea decrease.</b>	352	NA
	Tetrabenazine	Catecholamine-depleting agent	156	537	No neuroprotective or beneficial symptomatic effects	273	NCT00277602
	Clozapine	D2 receptor antagonist	8	63	<b>Improvement in chorea intensity.</b> No improvement in functional capacity or other clinical features	272	NA
	Tiapride	D2 receptor antagonist	0.7	30	<b>Improvement in chorea</b>	354	NA
	Amantadine	NMDA-receptor antagonist	12	84	<b>Improvement in chorea</b>	353	NA
	Remacemide hydrochloride*	NMDA receptor antagonist	4.4	33	<b>Little beneficial effects in chorea, only in neuroleptic naïve patients</b>	355	NA
			4	22	No significant effect in involuntary movements	367	NA
			9	29	<b>Significant improvement in chorea and motor symptoms</b>	356	NA
		4	24	<b>Only palliative benefit</b>	357	NCT00001930	
		2	24	No significant effect in chorea symptoms	368	NA	
		5	31	<b>Trend toward improvement in chorea with 200mg/day</b>	358	NA	

Continued

Table III. Continued

PolyQ disease	Therapeutic molecule	Mechanism of action	Treatment duration (weeks)	N	Overall efficacy	Ref.	Clinical trial code
	Remacemide hydrochloride and Coenzyme Q10 Donepezil*	NMDA receptor antagonist and antioxidant Acetylcholinesterase inhibitor	120	347	No significant effect in TFC	225	NA
	Baclofen* Modafinil Mavoglurant (AFQ056)	GABA agonist Analeptic Selective metabotropic glutamate receptor 5 antagonist	168 3 4	30 60 20 42	No significant effect in chorea, cognition, and quality of life No significant effect in TFC <b>Improvement in alertness</b> but not in cognition or mood No significant effect in chorea symptoms	369 370 359 371	NA NA NA NCT01019473
	PBT2 (8-hydroxyquinoline analog)*	metal-protein interaction-attenuating compound	26	109	No significant effect in cognition	372	NCT01590888
<b>SBMA</b>	Leuporelin*	Gonadotropin-Releasing Hormone (GnRH) analogue	48	199	No significant effects on swallowing function	373	NA
	Dutasteride	Dual 5- $\alpha$ reductase inhibitor	104	50	No significant effect on the progression of muscle weakness	374	NCT00303446
<b>SCA2</b>	Lithium Carbonate	Anti-psychoctic	48	20	Non-significant differences on the SARA, and for brain volume change; significant reduction in the BDI-II	375	NCT00998634
<b>SCA1,2,6</b>	Dalfampridine	Potassium channel blocker	10	20	Unreliable or uninterpretable data	NPP	NA
<b>Several SCAs</b>	Riluzole	Glutamate release inhibitor	52	60	<b>Improvement in motor function</b> given by decreased SARA score	376	NCT01104649
<b>MJD/SCA3</b>	Dalfampridine	Potassium channel blocker	10	20	Unreliable or uninterpretable data	NPP	NCT01811706
	Varenicline (Chantix)	$\alpha$ 4 $\beta$ 2 neuronal nicotinic acetylcholine receptor agonist	8	20	<b>Improvement of axial symptoms and rapid alternating movements.</b>	360	NCT00992771
	Lithium carbonate	Anti-psychoctic	48	62	No significant effect in NESCA score; minor reduction of progression in PATA speech-rate, non-dominant Click test, SCAFI and Composite Cerebellar Functional Score	377	NCT01096082
	Trimethoprim and sulfamethoxazole (Bactrim)	Antibiotic	24	22	No significant effect in movement scores and visual system	378	NA
	Valproic acid	histone deacetylase inhibitor	12	36	<b>Improvement in locomotor function</b> given by the decrease in global SARA score	255	NA

Positive effects are highlighted in bold.

\*Clinical trials based on previous preclinical data.

N, number of patients (treatment and placebo); MMSE, Mini-Mental State Examination; UHDRS, Unified Huntington's Disease Rating Scale; NPI, neuropsychiatric inventory; NARI, noradrenaline reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; NPP, no publication provided (trials.gov); NESCA, Neurological Examination Score for the Assessment of Spinocerebellar Ataxia; SCAFI, Spinocerebellar Ataxia Functional Index; SARA, Scale for the Assessment and Rating of Ataxia. BDI-II, Beck's depression inventory. NA, not accessible through public databases.

trials and with adequacy of models; extensive validation of findings by different labs and in more than one model may be demanded in the future to increase the probability of success in humans. Additionally, the correct study design and statistical measures should be used, as well as the right dosages, for each animal model so that results can be more rigorously translated to patients.

The vast majority of the clinical trials in polyQ diseases have been performed for HD, as depicted in Table III, which is the most common of these disorders. However, in the case of positive outcomes, some of the compounds may provide good candidates to test in other polyQ diseases. Nevertheless, the design and success of polyQ clinical trials constitute a challenge due to the particularities of these diseases, namely, the following: (i) highly variable clinical presentation and slow progression of symptoms; (ii) lack of reliable and specific targetable biomarkers; (iii) small number of patients; and (iv) high costs of clinical trials in relation to the investment for rare diseases. In fact, as mentioned above, the majority of clinical trials performed up to now have failed or produced slight success. Therefore, it is crucial to critically analyze the results obtained so far, so that we are able to design improved trials in the future and increase the likelihood of success. Some issues regarding patient risks and side effects, study duration, end-point sensitivity, availability of biomarkers of the disease(s), sample size, trial rationale and preclinical study results, pharmacological and methodology questions, and clinical trial design could underlie the current overall lack of positive results. On the other hand, despite the considerable advances regarding our knowledge of the genetics and pathophysiology of polyQ diseases in the past decade, the field is still lacking relevant biomarkers of the disease onset and progression, which must be also validated during RCTs along with clinical outcomes. Improving the reliability and validity of performance measures of the composite rating scales, neuroimaging studies and/or blood-based transcriptomic studies are major investments that should be made in the field, even though they may only bear results in the medium term. Finally, a more effective approach to early phase clinical trials is required to speed up the identification of suitable drugs for these diseases.

While no overall effective treatment for polyQ diseases is available, physical and/or palliative therapies to maintain the individual's independence in all environmental contexts should be implemented for as long as possible. The working goal of this kind of therapy is to establish strategies to optimize sensorial information, to improve cognition through different activities, enhance communication, increase the quality and control of movement in different body postures, improve approaches for an independent gait, exercise against resistance, and improve hypotonia and motor control and speech therapy.<sup>382-385</sup> There is also a need for these approaches to be studied using RCT experimental designs, and perhaps their synergistic effect with the above-mentioned candidate drugs should also be explored. In fact, in a very good example of such a study, Miyai et al. reported short-term evident beneficial effects of intensive rehabilitation in patients with degenerative cerebellar disease.<sup>386</sup> Even small steps may represent a great help and motivation leading to an improvement and sustainable quality of life for patients with polyQ diseases and their families.

## **ACKNOWLEDGMENTS**

We acknowledge Dr. Andreia Neves-Carvalho for the help in figure construction and design. This work was co-financed by the Project ON.2 SR&TD Integrated Program (NORTE-07-0124-FEDER-000021), co-funded by North Portugal Regional Operational Program (ON.2-O Novo Norte), under the National Strategic Reference Framework, through the European Regional Development Fund (ERDF) and also supported by Fundação para a Ciência e Tecnologia through the project POCI-01-0145-FEDER-016818 (PTDC/NEU-NMC/3648/2014).

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