Biologics: Targets and Therapy

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REVIEW

Emerging Therapies for Huntington's Disease – Focus on N-Terminal Huntingtin and Huntingtin Exon 1

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Abstract: Huntington's disease is a devastating heritable neurodegenerative disorder that is caused by the presence of a trinucleotide CAG repeat expansion in the *Huntingtin* gene, leading to a polyglutamine tract in the protein. Various mechanisms lead to the production of N-terminal Huntingtin protein fragments, which are reportedly more toxic than the full-length protein. In this review, we summarize the current knowledge on the production and toxicity of N-terminal Huntingtin protein fragments. Further, we expand on various therapeutic strategies targeting N-terminal Huntingtin on the protein, RNA and DNA level. Finally, we compare the therapeutic approaches that are clinically most advanced, including those that do not target N-terminal Huntingtin, discussing differences in mode of action and translational applicability.

Keywords: Huntingtin, N-terminal fragments, proteolysis, aberrant splicing, exon1 fragment, Huntington's disease therapeutics

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder with an estimated prevalence of up to 9 per 100,000 in the USA, Canada, Oceania, and Western Europe.[1,2] HD is caused by a CAG (cytosine, adenine, and guanine) repeat expansion in exon 1 of the *Huntingtin* (*HTT*) gene, resulting in the translation of a mutant Huntingtin protein harboring a toxic polyglutamine (polyQ) stretch at its amino (N) terminus. Gene carriers with repeats between 36 and 39 CAG show incomplete penetrance, while repeats of 40 and more triplets lead to fully penetrant disease. The age of onset is inversely correlated with the CAG repeat length, with an average age of onset of 35–44 years. HD is characterized by motor, cognitive and psychiatric symptoms and is ultimately fatal, with a median survival of 15–18 years after onset. About 5–10% of HD patients show disease onset before 20 years of age, in which case it is called juvenile HD. Juvenile HD has a different clinical presentation compared to adult onset HD, characterized by symptoms such as severe mental retardation, speech and language delay, as well as more pronounced motor and cerebellar symptoms and overall more rapid disease progression.[3]

Apart from the inherited CAG length, several genetic modifiers have been identified that are associated with age of onset. Many of these modifiers point towards an important role for somatic instability: the process in which the CAG repeat within cells expands over time. Within the *HTT* locus, a strong genetic modifier is whether or not a CAA (cytosine, adenine, and adenine) interruption is present at the 3' end of the CAG repeat. Similar to CAG triplets, CAA encodes for glutamine, thus resulting in the same polyQ stretch. Nonetheless, alleles that lack this CAA interruption were found to be more prone to somatic expansion and showed decreased age of onset, while the presence of an additional CAA interruption was found to delay both somatic expansion and age of onset.[4,5] Moreover, many of the identified *trans*-acting genetic modifiers, such as FANCD2 And FANCI Associated Nuclease 1 (FAN1) and MutL Homolog 1 (MLH1), are involved in DNA mismatch repair and influence somatic instability of the CAG repeat.[5,6]

Although HD was initially thought to be mainly a protein toxic gain-of-function disorder, it is likely that protein loss-of-function also plays a role, as reviewed elsewhere,[7–10] and there is increasing evidence for the involvement of

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other disease mechanisms, such as repeat-associated non-AUG dependent (RAN) translation and RNA toxic gain-of-function, also reviewed previously.[11–13] Still, little is known regarding the relative contribution of each of these pathogenic mechanisms to the disease (Figure 1).

HTT is known to be essential for embryonic development, as demonstrated by the fact that knockout mice are embryonically lethal, and also appears to play a role in later stages of development and life, as reviewed by Kaemmerer and Grondin.[10] There is, however, no clear consensus on the level of wild type HTT (wtHTT) that is required for its normal function, as this is likely to depend on many factors, including age and tissue/brain region. wtHTT is involved in many important cellular processes, including endocytosis and vesicular trafficking, cell division, autophagy and transcriptional regulation (reviewed by Saudou and Humbert)[9] which may all be impacted by a loss of wtHTT function in HD.

Compelling evidence for the involvement of RNA-mediated toxicity was provided by Sun et al, who found that even in the absence of translation, there was still repeat-length dependent toxicity of 5' *HTT* mRNA as well as full-length *HTT*.[14] RNA toxic gain-of-function is caused by the interaction between RNA-binding proteins (RBPs), such as Muscleblind like splicing regulator 1 (MBNL1) and Pre-mRNA processing factor 8 (PRPF8), and the secondary structure formed by the expanded CAG repeat in the mRNA, affecting the splicing of a range of transcripts.[15,16] This interaction appears to be dependent on the purity of the CAG repeat (ie, the absence of CAA interruptions), as Mbnl1 was found to be recruited to nuclear foci in the novel BAC-CAG mouse model, which has an uninterrupted repeat, but not in the BACHD model, which harbors an interrupted repeat.[17]

Finally, the presence of the expanded CAG repeat has also been shown to induce repeat-associated non-AUG dependent (RAN) translation, which leads to the production of homopolymers other than polyQ that may also negatively impact cell function. RAN translation products have been detected in the affected brain regions of patients, as well as in N171-82Q mice and a *C. elegans* model.[18,19] However, the actual contribution of RAN translation products to HD is not clear, as, for example, no RAN toxicity was observed in HD140Q knock-in mice.[20]

The expanded polyQ-containing mutant HTT (mHTT) protein has been shown to interact aberrantly with a variety of proteins, including transcriptional regulators such as RNA polymerase II subunit A (POLR2A), Tumor protein p53, Mouse double minute 2 (MDM2), CREB-binding protein (CBP) and Heat shock protein 70 (HSP70), cell cycle regulators like Ras homolog enriched in brain (Rheb) and mammalian target of rapamycin (mTOR), and cytoskeleton proteins such as actin and neurofilament light (NF-L). These aberrant interactions result in a complex and widespread molecular pathology, affecting many essential processes in the cell, including DNA damage repair, transcriptional regulation, mitochondrial function and apoptosis.[21–25] Importantly, premature polyadenylation of the pre-mRNA as well as proteolytic cleavage of HTT protein lead to the production of a variety of HTT fragments, and there is ample evidence that such fragments, especially the short N-terminal species, are more toxic than the full-length mHTT protein.[26–35] In order to make tailored therapeutics towards the short toxic fragments, a good understanding of the mechanisms leading to their formation is

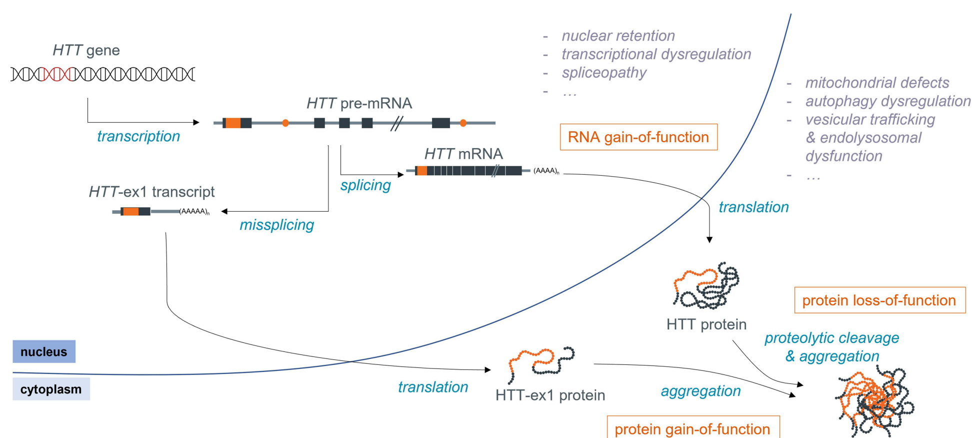

Figure 1 Schematic overview of the molecular pathogenesis of HD.

needed. In this review, we therefore focus on how toxic N-terminal HTT protein species are produced and how they are linked to toxicity, as well as on therapeutic strategies that are capable of reducing these fragments.

Production of Toxic N-Terminal HTT Protein Species

N-terminal HTT protein fragments are mainly produced through two distinct processes: proteolytic cleavage and premature polyadenylation (see Figure 2 and Table 1).

Proteolytic Cleavage

Caspase Cleavage

The group of Michael Hayden first showed that HTT could be cleaved proteolytically by apopain (caspase-3) in a repeat-length dependent manner.[36] This was confirmed in a follow-up study, in which they mapped one of the caspase-3 cleavage sites to D513 and another site C-terminally of amino acid (aa) 548. Furthermore, two caspase-1 cleavage sites were identified in the first 548 aa. In contrast to their previous work with truncated HTT, the authors found no repeat-length dependence of cleavage efficiency of full-length HTT.[37] In a third study, the authors were able to map the second caspase-3 cleavage site to D552, and further identified a caspase-6 cleavage site at D586.[38] More recently, Martin et al recently identified yet another caspase cleavage site at D572, which was shown to be cleaved by caspase-1 and caspase-2.[39]

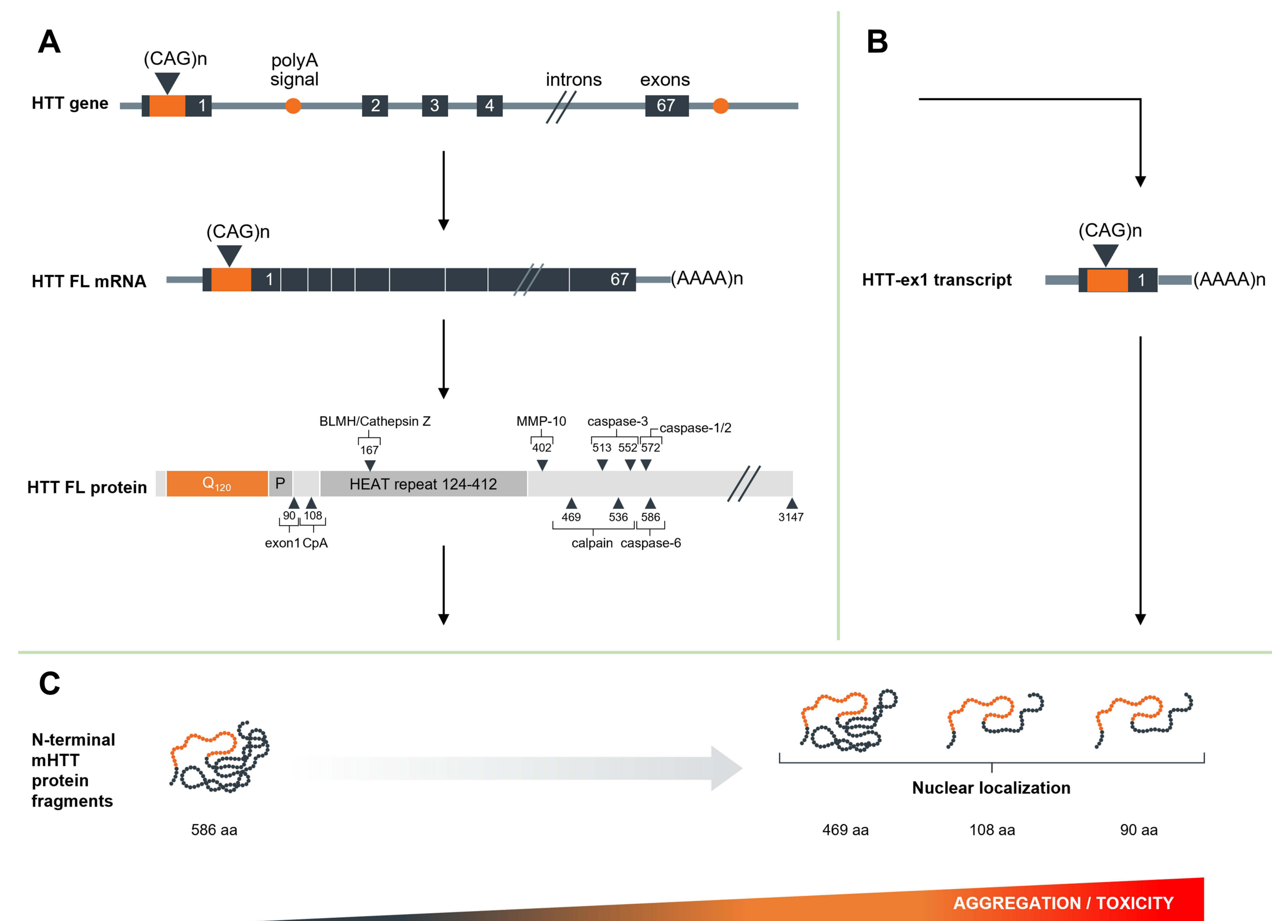

Figure 2 Schematic overview of production of N-terminal HTT protein. (**A**) Regular splicing, overview of the resulting mRNA and full-length protein and the identified proteolytic cleavage sites. (**B**) Alternative splicing and premature polyadenylation and resulting transcript. (**C**) Resulting protein species and propensity for nuclear entry, aggregation and toxicity.

Table 1 Overview of Proteolytic Cleavage Sites

Cleavage Site	Enzyme	References	Notes
90–105 (cp-1)	Undetermined	Ratovitski et al 2007[46]	
104–114 (cp-A)	Aspartic endopeptidases?	Lunkes et al 2002;[43] Tebbenkamp et al 2012[45]	Tebbenkamp et al were unable to inhibit formation of this product, suggesting that it may generated by a novel protease and not aspartic endopeptidases ('?': conflincting evidence)
63–111	Calpain	Sun et al 2002[42]	
146–214 (cp-B)	Aspartic endopeptidases?	Lunkes et al 2002[43]	
R167	Cysteine endopeptidases including bleomycin hydrolase and cathepsin Z	Ratovitski et al 2009;[162] Ratovitski et al 2011[163]	
402	MMP-10	Miller et al 2010[161]	
465, 469	Calpain	Gafni et al 2002[41]	
473	Calpain	Gafni et al 2002[41]	
D513	Caspase-3	Wellington et al 1998;[37] Wellington et al 2000[38]	
536, 540	Calpain	Gafni et al 2002[41]	
D552	Caspase-3, caspase-2?	Wellington et al 2000[38]	
D572	Caspase-1	Martin et al 2019[39]	
D586	Caspase-6	Wellington et al 2000[38]	

Calpain Cleavage

Both full-length and N-terminal caspase-cleavage products of HTT were found to be substrates for cleavage by calpains.[40–42] Four calpain cleavage sites have been mapped, at aa 437, 465/469 and 536/540[41] and between aa 63–111,[42] calpain cleavage efficiency appears to be positively correlated with repeat length.[41,42] Furthermore, it was shown that calpain levels, and in particular the active form, were increased in the caudate of HD patients compared to controls.[41]

Other Proteases/Unidentified Mechanisms

Next to caspase and calpain generated fragments, various other cleaved HTT products have been described. Lunkes et al identified two N-terminal HTT fragments, cp-A and cp-B, which appeared to be generated in transfected NG108 cells through cleavage by aspartic endopeptidases. The C-terminus of HTT cp-A fragment was mapped between aa 104–114. N-terminal fragments with the same immunogenic properties were identified in nuclear inclusions in post mortem frontal cortex of HD patients.[43]

Similarly, Schilling et al identified an N-terminal fragment ending between aa 90–115 in post mortem tissues from HD patients and N171-82Q mice, as well as in transfected HEK293 cells.[44] Further investigation in a HEK293 cell model revealed that short, HTT cp-B-like fragments were efficiently processed to HTT cp-A-like fragments, while longer HTT fragments proved to be inefficient substrates. The C-terminus of the HTT cp-A-like fragments was mapped between aa 105 and 115–124. Although similar in size to the fragment described by Lunkes et al, inhibition of aspartyl proteases did not affect the formation of the cp-A-like fragment, and the authors were unable to identify any protease that generates

these HTT cp-A-like fragments, suggesting that i) the fragments are not the same or ii) that the cp-A-like fragment described by Schilling et al is the same fragment but generated by a novel protease, which may be cell-type dependent.[45] Ratovitski et al identified two N-terminal fragments (HTT cp-1 and cp-2) in PC12 and HEK293 cells expressing full-length HTT with 21Q or 126–153Q or a truncated N1212 HTT fragment with 15Q or 138Q.[46] These fragments were similar in size to the previously described HTT cp-A and cp-B fragments but were not affected by inhibition of aspartic endopeptidases. In addition, they were not affected by deletion of aa 105–114. In combination with the epitope mapping, this narrowed the C-terminus of the HTT cp-1 fragment down to between aa 90 and 105, shorter than the cp-A and cp-A-like fragments described by Lunkes et al[43] and Schilling et al[44,45] Based on the absence of identified proteases and on the fragment length, we speculate that the generation of these fragments could involve aberrant splicing (see Aberrant Splicing and Premature Polyadenylation), although this would require further investigation.

Finally, Landles et al showed fourteen different N-terminal HTT protein isoforms (fragments 1–14) in brain tissue from HdhQ150 KI mice, the three shortest of which (fragments 12–14) were specific to mHTT.[33] Some of these fragments could be linked to specific proteolytic cleavage events: fragment 7 terminated at a novel calpain cleavage site between aa 510–654, fragment 8 appeared to correspond to the D586 caspase-6 cleavage product, fragment 9 was likely produced by cleavage at calpain site 536 and fragment 10 by caspase cleavage at D513. Lastly, fragment 13 was determined to correspond to HTT-ex1.

In summary, many different proteases have been found to act on mHTT and wtHTT, generating N-terminal and C-terminal HTT fragments. The availability of antibodies that can recognize these fragments, as well as the possibility to specifically inhibit certain proteases, have allowed mapping of various fragments, albeit with variable resolution. Nonetheless, for multiple fragments, the mechanisms of production remain to be identified.

Aberrant Splicing and Premature Polyadenylation

Besides proteolytic cleavage, there are other mechanisms that lead to the generation of toxic N-terminal mHTT fragments. Sathasivam et al showed that incomplete splicing of intron 1 leads to the production of a short premature polyadenylated *HTT*-ex1 transcript in various HD mouse models and that this *HTT*-ex1 can be translated into a 90 aa N-terminal HTT-ex1 protein (based on 23Q). *HTT*-ex1 transcript was also found to be expressed in HD patient fibroblasts and cortex.[47] In a follow-up study, Neueder et al confirmed that the HTT-ex1 transcript can be detected in patient-derived fibroblasts, as well as HD patient cerebellum, sensory motor cortex and hippocampus, with the highest expression levels measured in juvenile HD patient tissues.[48] The *HTT*-ex1 transcript has also been detected by RNA-sequencing in various HD mouse models, including BACHD, BAC-CAG and HdhQ111.[17] Both in vitro and in patient-derived tissues, the production of the *HTT*-ex1 transcript appears to be positively correlated with CAG repeat length, showing much higher expression in cells and tissues derived from juvenile HD patients.[48,49]

The current hypothesis is that HTT-ex1 formation is influenced by a combination of sequestration of spliceosome components such as U1 snRNP at the CAG repeat, leading to less efficient splicing of exon 1 to exon 2, and a reduced transcription rate, which leads to longer exposure of the cryptic polyA site in intron 1. Although the Bates group initially found evidence for the involvement of Serine and Arginine Rich Splicing Factor 6 (SRSF6) in *HTT*-ex1 formation,[47,49] they later found that the silencing of Srsf6 in HD mouse models did not affect HTT-ex1 formation.[50] It has therefore been hypothesized that multiple RNA-binding proteins may be involved in the missplicing of HTT-ex1.[12] Regardless of the exact mechanisms involved, aberrant mHTT splicing is CAG repeat length dependent, suggesting that HTT-ex1 formation and associated toxicity would increase as somatic instability progresses in HD[48] and that interventions targeting repeat expansion and HTT-ex1 may have therapeutic advantage.

Properties of N-Terminal Protein Species

Consistently accumulating evidence indicates that small N-terminal fragments containing extended polyQ tracts significantly contribute mHTT cellular mislocalization, aggregation and toxicity. Initial studies by the Ross group showed that transfection of N2a or HEK293 cells with full-length HTT with either 23Q or 82Q, or of truncated HTT N171-18Q or N63-18Q resulted in a diffuse cytoplasmic localization of the protein. In contrast, transfection with N171-82Q or N63-82Q led to more punctate labeling in both cytoplasm and nucleus, with the short N63-82Q construct showing the most

prominent nuclear localization.[51] The Hayden group found similar results, showing that N-terminal fragments of 427, 548 or more aa formed mainly perinuclear aggregates, while fragments up to 224 aa showed both cytoplasmic and nuclear aggregates. Furthermore, they found that pathogenicity depended both on repeat length and on fragment size.[26,27]

Barbaro et al found that, in *Drosophila*, shorter N-terminal fragments were more toxic and more prone to aggregate, with HTT-ex1 being by far the most toxic species.[28] In mice, the R6/2 model that expresses only HTT-ex1 is by far the most swiftly progressing HD mouse model,[52,53] while conditional suppression of HTT-ex1 has been shown to be neuroprotective.[54] Recent in vitro studies by the Lashuel group confirm these results and further extend the findings by showing that the polyQ and Nt17 domains of HTT-ex1 synergistically modulate the aggregation propensity of HTT-ex1, with a key role of the Nt17 domain in regulating HTT-ex1 aggregation dynamics and subcellular localization and toxicity.[34]

There is conflicting evidence with regard to the pathogenicity of nuclear and cytoplasmic mHTT. Some groups have reported evidence that nuclear localization is required for toxicity. For example, the Greenberg group showed that adding a nuclear export signal to a N171 HTT fragment blocked its toxicity in transfected striatal neurons.[55] In contrast, the Hayden group reported that neither the addition of a nuclear localization signal to a N548 HTT fragment nor the addition of a nuclear export signal to a N151 fragment altered the toxicity of those fragments, suggesting that both the nucleus and the cytoplasm are sites of HD toxicity.[56] Trushina et al found that nuclear entry of mHTT only occurred after commitment of a cell to cell death. Therefore, the authors argue that nuclear mHTT localization may not be the primary event leading to toxicity.[57]

Intranuclear and neuropil aggregates have been observed in most HD animal models,[17,30,31,58–63] and the presence of aggregates containing N-terminal HTT fragments has also been confirmed in patient brains by multiple groups.[40,64,65] However, various groups have shown that it is not the insoluble aggregates or inclusion bodies, but rather the soluble oligomers that are the more toxic species.[66–69] In fact, some groups have found evidence that the formation of intranuclear inclusions may be protective,[55,70,71] as reviewed by Arrasate and Finkbeiner.[72] Mechanistically, this may be explained by the fact that soluble mHTT-ex1 oligomers have more aberrant protein interactions than insoluble aggregates and inclusions.[73] Importantly, the length of N-terminal protein species and the associated sequence context, as well as post-translational modifications, also appear to play an important role in the aggregation process.[35,74,75] For more in-depth reviews on the role of post-translational modifications, we redirect elsewhere.[76,77]

Therapeutic Strategies to Reduce N-Terminal HTT and HTT-ex1

Various approaches have been investigated to therapeutically lower the expression or reduce the toxicity of the mutant HTT protein. The proteolytic cleavage pathway can be targeted to reduce the formation of N-terminal mHTT protein species. Furthermore, the N-terminal part of the protein can be targeted to reduce aggregation and/or increase clearance of mHTT. Finally, mHTT can be targeted at the transcript or gene level. Here, we will focus on approaches that are able to target not only full-length HTT but also HTT-ex1 and other N-terminal mHTT species, considering their potential therapeutic advantage (see Table 2).

Reducing Proteolytic Cleavage

Caspase inhibition has been shown to reduce the proteolytic cleavage of mHTT and to improve the HD phenotype in BACHD[78] and HdhQ111 mice.[79] These results are backed up by earlier studies, where mutation of caspase-6 cleavage sites slowed down disease progression in YAC128 mice.[80] However, it is not clear to what extent the protective effects are due specifically to the reduction of N-terminal mHTT species, rather than a general protective effect of caspase inhibition, as caspase inhibition was also protective in R6/2 and malonate models of HD, which do not express caspase-cleavable mHTT.[81–83]

Using a different approach, Evers et al showed that removal of the caspase-6 cleavage site by antisense oligonucleotide (ASO)-mediated skipping of (part of) exon 12 led to reduced levels of the N568 fragment in vitro and in vivo in wild type and YAC128 mice.[84,85] Except for the absence of astrogliosis, no data are available regarding phenotypic effects of this ASO treatment.

None of these approaches have yet successfully been translated into the clinic, and although all may potentially decrease the formation of toxic mHTT fragments and have the potential of allele-specificity, mechanisms of RNA-associated toxicity would not be addressed.

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Table 2 Overview of Studies Targeting HTT Protein

References	Therapeutic Modality	Name	In vitro Models	In vivo Models
Caspase inhibition				
Leyva et al 2010[79]	Peptidomimetic	Novel pan-caspase inhibitors	HEK293T overexpressing myc-tagged full-length HTT (23 or 148Q); STHdh cells; rat striatal and cortical neurons expressing N90 (73Q)	
Aharony et al 2015[78]	Peptide	ED11	Striatal extracts from BACHD mice; HEK293 cells overexpressing N1212 (15Q); PC12 cells with inducible 145Q-mHTT	BACHD
Evers et al 2014[84]	ASO	QRX-704	HD patient-derived fibroblasts; murine C2C12 cells	WT mice
Klein et al 2018[85]	ASO	QRX-704	HD patient-derived fibroblasts; HD iPSC-derived neurons; COS7 cells	YAC128 mice
Decreasing aggregation and increasing mHTT-ex1 clearance				
Chaudhary et al 2015[86]	Aptamers	mHtt2.2.18, mHtt2.2.47, mHtt2.3.42	Recombinant HTT-ex1 proteins; yeast expressing HTT-ex1	
Patel et al 2018[87]	Aptamers	mHtt2.2.18, mHtt2.3.42	Yeast expressing HTT-ex1	
Southwell et al 2008[90]	Intrabodies	Happ1, Happ3, MW7, VL12.3	HEK293 cells overexpressing mHTT-ex1; rat brain slices transfected with mHTT-ex1; ST14A cells overexpressing mHTT-ex1	
Southwell et al 2009[91]	Intrabodies	Happ1, VL12.3		Lentiviral mouse model; R6/2 mice; N171-82Q mice; YAC128 mice; BACHD mice
Southwell et al 2011[92]	Intrabodies	Happ1, Happ3, MW7, VL12.3	HEK293 cells overexpressing mHTT-ex1; ST14A cells overexpressing mHTT-ex1	
de Genst et al 2015[93]	Intrabodies	scFv-C4	Recombinant HTT-ex1 proteins	
Butler et al 2014[89]	Intrabodies	scFv-C4, VL12.3	ST14A cells overexpressing mHTT-ex1	R6/1 mice
Lecerf et al 2001[94]	Intrabodies	scFv-C4	COS-7, BHK-21 and HEK293 cells overexpressing mHTT-ex1 constructs	
Murphy and Messer 2004[95]	Intrabodies	scFv-C4	Murine brain slices transfected with HTT-ex1	
Wolfgang et al 2005[96]	Intrabodies	scFv-C4		*Drosophila* expressing mHTT-ex1
Miller et al 2005[97]	Intrabodies	scFv-C4	BHK-21, HEK293 and ST14A cells overexpressing HTT-ex1; STHdh cells	
Snyder-Keller et al 2010[98]	Intrabodies	scFv-C4		R6/1 mice

(*Continued*)

Table 2 (Continued).

References	Therapeutic Modality	Name	In vitro Models	In vivo Models
Wang et al 2008[99]	Intrabodies	scFv-EM48	HEK293 cells, rat primary cortical neurons and PC12 cells overexpressing HTT-ex1	R6/2 mice; N171-82Q mice
Amaro and Henderson 2016[100]	Intrabodies	INT41, Happ1	HEK293T cells overexpressing HTT-ex1; PC12 cells with inducible full-length HTT	R6/2 mice
Bauer et al 2010[102]	Fusion protein	QBP1-HSC70	Neuro2a cells expressing HTT-ex1	R6/2 mice; HD190Q-EGFP mice
Clift et al 2017[103]	Fusion protein	3B5H10-TRIM21	3T3 cells and oocytes overexpressing partial HTT-ex1 (aa 8–57)	
Butler et al 2011[104]	Fusion protein	scFv-C4-PEST	ST14A cells overexpressing HTT-ex1	
Ghosh et al 2021[105]	Endogenous protein	Praja1 ubiquitin ligase	HEK293T and N2A cells overexpressing HTT-ex1	
Hegde et al 2020[106]	Endogenous protein	TBK1	Recombinant HTT-ex1; HEK293 cells overexpressing HTT-ex1; rat primary striatal neuronal cells; mouse primary striatal neurons transfected with HTT-ex1 or N586	*C. elegans* overexpressing N513 15Q or 128Q
Aladdin et al 2020[107]	Endogenous protein	Blm10/ PA200	Recombinant HTT-ex1; yeast and SH-SY5Y cells overexpressing HTT-ex1	
Galyan et al 2022[108]	Small molecule	GLYN122	PC12 cells with inducible mHTT-ex1	R6/2

Decreasing Aggregation and Increasing mHTT-ex1 Clearance

Aptamers

Aptamers are single-stranded oligonucleotides that, through their tertiary structure, can interact with target molecules such as proteins. The Roy lab identified aptamers that bind specifically to mHTT with 51 or 103Q but not wtHTT with 20Q.[86,87] The selected aptamers were shown to inhibit aggregation of recombinant mHTT-ex1 in cell-free assays and in yeast, as well as reducing oxidative stress and mitochondrial dysfunction.[86] To our knowledge, this approach has not yet been tested in vivo.

Intrabodies

Various antibodies have been expressed intracellularly as "intrabodies" to target the N-terminus of HTT. In vivo, such intrabodies are delivered using viral vectors. An excellent review on the use of intrabodies in various neurodegenerative diseases was written by Messer and Butler.[88]

Two groups of intrabodies have been tested most extensively (see Figure 3): those that bind to the N-terminus of HTT (V_L12.3, scFv-C4) and those that recognize the proline-rich regions (PRRs) in HTT-ex1 (MW7, Happ1, Happ3, INT41). In addition, there is some literature about polyQ-binding intrabodies (MW1, MW2) and a more C-terminal intrabody derived from EM48 (scFv-EM48).

Southwell et al showed that intrabodies that bind to the PRR, ie, MW7, Happ1 and Happ3, increase the turnover of mHTT-ex1 overexpressed in vitro. V_L12.3, an intrabody that binds to the N-terminal 17 aa of HTT, did not affect turnover, but did increase the nuclear localization of mHTT-ex1.[90] In vivo, the PRR-binding Happ1 was shown to be beneficial in five different HD mouse models. In contrast, V_L12.3, while effective in a lentiviral HD model, was

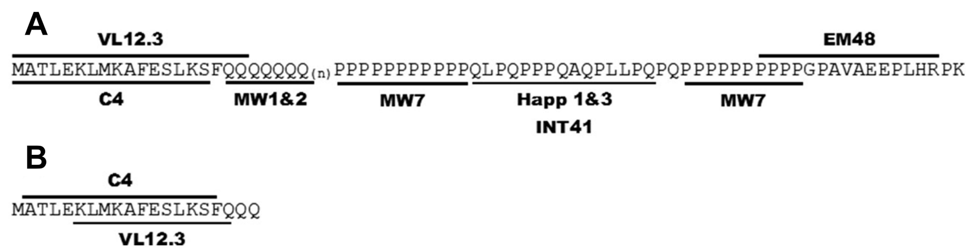

Figure 3 Anti-HTT Exon 1 intrabodies. (**A**) Antigens used to select the published anti-HTT intrabodies. (**B**) Specific binding identified by crystallography for scFvC4 and V_L12.3.

Notes: Reproduced from Messer A, Butler DC. Optimizing intracellular antibodies (intrabodies/nanobodies) to treat neurodegenerative disorders. *Neurobiol Dis*. 2020;134 (October 2019):104619. doi: 10.1016/j.nbd.2019.104619 under Creative Commons BY-NC-ND 4.0.[88]

ineffective in YAC128 mice and had a detrimental effect in R6/2 mice.[91] The authors later showed that the increased turnover mediated by the PRR-binding intrabodies is dependent on a calpain-chaperone-mediated autophagy-dependent mechanism and that this process is blocked by V_L12.3,[92] explaining the detrimental effects of V_L12.3.

Although scFv-C4 also binds to the N-terminus of HTT,[93] its predominant cytoplasmic localization appears to protect from the detrimental effects observed for V_L12.3.[89] The scFv-C4 intrabody was shown to have beneficial effects in various HD models, including in vitro models, Drosophila and different mouse models.[94–98]

Two additional intrabodies have been investigated: scFv-EM48 and INT41. Like Happ1, scFv-EM48, which binds just C-terminally to the second PRR, was shown to increase turnover of mHTT, and improved motor function of N171-82Q mice.[99] INT41, an intrabody that recognizes the same epitope as Happ1, but which has enhanced cytoplasmic solubility, was shown to improve cognitive function in female R6/2 mice.[100]

Engineered Molecules and Endogenous Proteins

In addition to the increased turnover induced by some of the intrabodies, the endogenous cellular machinery can be harnessed specifically to target proteins for degradation, using engineered proteins, peptides or small molecules. These can direct the protein of interest to the ubiquitin proteasome system, the autophagy-lysosomal pathway or chaperone-mediated autophagy. These approaches and their specific application in the context of HD have been extensively reviewed by Jarosińska and Rüdiger.[101]

Two such approaches specifically target the polyQ region. Bauer et al engineered a fusion molecule consisting of two copies of a polyQ-binding peptide (QBP1) and heat shock-cognate protein 70 (HSC70)-binding motifs to induce chaperone-mediated autophagy.[102] Clift et al co-expressed a polyQ-binding antibody (3B5H10) with TRIM21 in an approach that they call Trim-Away, to target mHTT for proteasomal degradation.[103] Additionally, Butler et al produced a fusion protein consisting of the scFv-C4 intrabody and a PEST motif to enhance proteasomal degradation of HTT-ex1.[104]

Several endogenous proteins have been described to enhance the turnover of mHTT, including Praja1 ubiquitin ligase,[105] TBK1[106] and Blm10/PA200.[107] Induction or overexpression of such proteins may represent a therapeutic strategy, although, so far, this notion is only supported by experiments in cellular, *Drosophila*, and *C. elegans* models. Additionally, specificity for mHTT has not been shown for any of these three proteins.

Finally, a small molecule that can bind to mHTT-ex1, called GLYN122, has been identified recently. GLYN122 was shown to reduce mHTT-ex1 aggregation in PC12 cells, as well as reducing mHTT in cortex and striatum of R6/2 mice after intraperitoneal injection.[108]

Targeting HTT-ex1 at the Transcript Level

Next to targeting the pathogenic protein species itself, the production of such proteins can also be inhibited by targeting the *HTT* mRNA. Many different approaches have been tested to this effect, including ASOs, siRNAs, shRNAs and miRNAs (Table 3). Again, we only focus on those strategies that target *HTT*-ex1. Broadly speaking, the *HTT*-ex1 mRNA targeting approaches can be divided into those that target the expanded CAG repeat, and those that target other regions of *HTT*-ex1. In addition, some other approaches have been described.

Table 3 Overview of Studies That Evaluated Therapeutic Approaches Targeting *HTT* at the RNA Level

References	Therapeutic Modality	In vitro Models	In vivo Models
Targeting CAG repeat			
Hu et al 2009[109]	ASO, siRNA	HD patient-derived fibroblasts, YAC128-derived primary medium spiny neurons	
Hu et al 2010[110]	siRNA	HD patient-derived fibroblasts	
Gagnon et al 2010[111]	ASO	HD patient-derived fibroblasts	
de Mezer et al 2011[112]	siRNA	HD patient-derived fibroblasts	
Fiszer et al 2011[113]	siRNA	HD patient-derived fibroblasts	
Yu et al 2012[115]	siRNA	HD patient-derived fibroblasts	HdhQ150 mice
Aiba et al 2013[116]	siRNA	HD patient-derived fibroblasts	
Liu et al 2013[117]	siRNA	HD patient-derived fibroblasts	
Monteys et al 2015[118]	miRNA	HEK293 cells overexpressing tagged full-length wtHTT and mHTT	Transgenic mice expressing tagged full-length wtHTT and mHTT
Fiszer et al 2016[114]	siRNA	HD patient-derived fibroblasts; STHdhQ7/Q111 and STHdhQ111/111 cells	
Batra et al 2017[140]	RNA-targeting Cas9	COS-M6 cells overexpressing a 80CAG construct	
Urbanek et al 2017[119]	ASO, siRNA	HD patient-derived fibroblasts	
Datson et al 2017[120]	ASO		R6/2 and Q175 mice
Ciesolka et al 2021[121]	siRNA	Inducible HEK293 model expressing HTT-ex1 16/98 CAG fused to luciferase; HD patient-derived fibroblasts; HD iPSC-derived neural progenitors	
Kotowska-Zimmer et al 2022[122]	shRNA, miRNA	HD patient-derived fibroblasts	YAC128 mice
Targeting other parts of HTT-ex1			
Boado et al 2000[125]	ODN	Inducible PC12 model expressing HTT-ex1-GFP with 25Q	
Chen et al 2005[126]	shRNA	HeLa, HEK293, DAOY cerebellar medulloblastoma cells	
Wang et al 2005[127]	siRNA		R6/2 mice

(*Continued*)

Table 3 (Continued).

References	Therapeutic Modality	In vitro Models	In vivo Models
Rodriguez-Lebron et al 2005[128]	shRNA	HEK293 cells expressing mHTT-ex1	R6/1 mice
DiFiglia et al 2007[129]	siRNA		AAV100Q mouse model
Denovan-Wright et al 2008[130]	siRNA, ribozyme	HEK293 cells expressing mHTT-ex1	R6/1 mice
Kordasiewicz et al 2012[131]	ASO		R6/2 mice
Rindt et al 2012[138]	Trans-splicing	HEK293, U2OS and DBRTG cells overexpressing a HTT minigene (exon1-3 with shortened introns); HEK293 cells; HD patient-derived fibroblasts	
Miniarikova et al 2016[123]	miRNA	HEK293T luciferase assay	Hu128/21 mice
Miniarikova et al 2017[124]	miRNA		Lentiviral rat model
Rindt et al 2017[139]	Trans-splicing	HEK293 cells; HD patient iPSC-derived neural stem cells and iPSC-derived neurons	
Evers et al 2018[132]	miRNA		tgHD minipigs
Caron et al 2019[133]	miRNA		Hu128/21 mice
Spronck et al 2019[134]	miRNA		Q175 mice and R6/2 mice
Keskin et al 2020[135]	miRNA	HD patient iPSC-derived neurons and astrocytes	
Valles et al 2021[136]	miRNA		tgHD minipigs
Spronck et al 2021[137]	miRNA		Wild type rats and NHP (GLP-tox)

Antisense Oligonucleotides and RNAi Agents

Many studies have tested ASOs or RNAi agents to target the CAG repeat.[109–122] In general, CAG-targeting confers preference towards the expanded allele, as this allows for binding of multiple molecules per mRNA.[111] Only a few studies included in vivo efficacy. Yu et al showed the efficacy of their siRNA in HdhQ150 mice.[115] Monteys et al used transgenic mice expressing tagged full-length wtHTT and mHTT, showing preferential silencing of mHTT.[118] Datson et al showed the efficacy of their CAG-targeting ASO in R6/2 and Q175 mice,[120] an ASO that is now further developed by Vico Therapeutics. Kotowska-Zimmer et al have shown that artificial miRNAs targeting the CAG repeat specifically reduced mHTT in YAC128 mice.[122]

A number of strategies that target other regions of *HTT*-ex1 have been described as well.[123–137] This approach would be expected to lower both wtHTT and mHTT. With the exception of Boado et al and Kordasiewicz et al, who used ASOs,

all of these studies utilized RNAi agents. Various groups have demonstrated efficacy of siRNA or shRNA in R6/1, R6/2 and AAV100Q mice.[127–130] uniQure's miRNA therapy has shown target engagement in the widest range of HD animal models, including Hu128/21, Q175 and R6/2 mice, lentiviral rat model and transgenic HD minipigs,[123,124,132–134,136] as well as a favorable safety profile in toxicity studies in rats and non-human primates.[137]

Other RNA-Targeting Approaches

A handful of studies described other approaches to *HTT* RNA-targeting. Rindt et al developed a method to induce trans-splicing, by which m*HTT* exon 1 is replaced with exogenous wt*HTT* exon 1 in the mRNA. Thus far, there is only in vitro proof of principle for this approach, and the efficiency is rather low, with 10–15% of trans-splicing observed even after extensive optimization.[138,139] Batra et al have developed an RNA-targeting Cas9 approach which targets the CAG repeat.[140] For HD, there is only in vitro evidence for this approach so far, but a similar approach targeting a CUG (cytosine, uracil, and guanine) repeat was shown to be effective in vivo in myotonic dystrophy type 1 mouse models.[141] This platform is being developed by Locanabio.

Finally, some small molecules have been described to bind to either *HTT*-ex1 or the CAG repeat, most notably furamidine, myricetin and a series of pyridocoumarin derivatives, reviewed elsewhere.[12] These compounds have been described to inhibit translation of *HTT*. However, specificity of such compounds is generally low, thereby increasing the chance of unwanted off-target effects.

Targeting HTT-ex1 at the DNA Level

Finally, several approaches that target the *HTT* gene have been described (Table 4).

Transcription can be prevented using zinc finger proteins (ZFPs) targeting the expanded CAG repeat.[142–144] This approach shows allele-selectivity for the expanded repeat and is currently being developed for the clinic by Sangamo and Takeda. Further, CRISPR-Cas9 genome editing approaches have been developed to either knock out *HTT* by inducing mutations or excise the region containing the CAG repeat. Several groups have shown in vitro and in vivo proof of principle using single guide RNAs directed to *HTT*-ex1 to induce *HTT* knockout.[145–148] Further, using a double guide RNA approach, various groups have shown that it is possible to excise the region containing CAG repeat.[149–154] The size of this region differs based on the chosen guide RNAs, with the first report by Shin et al deleting a large 44 kb region,[149] while the most precise excision was shown by Yang et al and Monteys et al, who deleted only the CAG repeat and small flanking regions.[150,151]

HTT Targeting Therapies in Clinical Development

Several HTT lowering therapies are either already in clinical trials or are close to entering the clinic. These therapies include different therapeutic modalities and mechanisms of action, each with distinct potential efficacy and safety profiles. Only the approaches in clinical trials or performing IND-enabling studies are covered here.

Two of the most advanced programs, the Phase III trial with the non-allele-specific *HTT* exon 36-targeting ASO tominersen (Roche) and the phase I/II trials with the allele-specific m*HTT*-associated single nucleotide polymorphism (SNP)-targeting ASOs WVE-120101 and WVE-120102 (Wave Life Sciences) were halted in 2021, as reviewed elsewhere.[155] Roche plans to design a new Phase II study with tominersen, for younger adult patients with lower disease burden (https://ir.ionispharma.com/news-releases/news-release-details/ionis-partner-evaluate-tominersen-huntingtons-disease-new-phase). Wave Life Sciences has now initiated a new trial with their novel product WVE-003, which targets another SNP and has improved chemistry (clinicaltrials.gov NCT05032196). These ASOs are administered repeatedly through intrathecal administration, which may explain some of the adverse events observed with tominersen, which was more pronounced in the cohort receiving more frequent administration.[155] Neither drug is expected to affect HTT-ex1 formation or RNA-mediated toxicity.

Novartis and PTC Therapeutics both have initiated Phase 2 clinical trials for their splicing modulators Branaplam (NCT05111249) and PTC518 (NCT05358717). These small molecules induce the inclusion of a pseudoexon between *HTT* exons 49 and 50, which leads to a premature stop codon and subsequent nonsense-mediated decay.[156,157] One of the main advantages is that these small molecules can be administered orally. Furthermore, the mechanism of action targets

Table 4 Overview of Studies Targeting the *HTT* Gene

References	Therapeutic Modality	Type	Target Region	In vitro Models	In vivo Models
Garriga-Canut et al 2012[142]	ZFP		CAG repeat	HEK293T cells overexpressing reporters with N-terminal region of HTT; STHdh cells; HD patient-derived mesothelial cell line	R6/2 mice
Agustin-Pavon et al 2016[143]	ZFP		CAG repeat		R6/1 mice
Zeitler et al 2019[144]	ZFP		CAG repeat	HD patient-derived fibroblasts; STHdh cells; HD patient ESC-derived neural stem cells and neurons	Q50 KI mice; R6/2 mice; zQ175 mice
Kolli et al 2017[145]	CRISPR-Cas9	Single guide	5' UTR or exon 1-intron 1 boundary	Mesenchymal stem cells derived from YAC128 mice	
Merienne et al 2017[146]	Self-inactivating CRISPR-Cas9	Single guide	Exon 1 just downstream of the start codon	HEK293T cells overexpressing N171-HTT-eGFP; primary murine cortical neurons and astrocytes; HD patient iPSC-derived neurons	Lentiviral mouse model; HD140Q-KI mice
Ekman et al 2019[147]	CRISPR-Cas9	Single guide	Exon 1 (5' and 3' of CAG repeat)	HEK293T cells overexpressing HTT-ex1-CFP	R6/2 mice
Powell et al 2022[148]	CRISPR-Cas13	Single guide	Exon 1 (5' and 3' of CAG repeat)	HEK293T cells overexpressing HTT-ex1-CFP	R6/2 mice
Shin et al 2016[149]	CRISPR-Cas9	Double guide; allele-specific	Deletion of 44 kb region spanning from promoter region to intron 3	Patient-derived fibroblasts, NSCs and iPSCs	
Yang et al 2017[150]	CRISPR-Cas9	Double guide	Deletion of part of exon 1 from just upstream to just downstream of the CAG repeat	HEK293 cells overexpressing HTT-ex1	HD140Q-KI mice
Monteys et al 2017[151]	CRISPR-Cas9	Double guide; allele-specific	Deletion of 1182 bp region from 3' end of promoter to 5' end of intron 1	HEK293 cells; HD patient-derived fibroblasts	BACHD mice
Dabrowska et al 2018[152]	CRISPR-Cas9 nickases	Double guide	Deletion of 107 bp in exon 1, from just upstream to downstream of the CAG repeat	HEK293T cells; HD patient-derived fibroblasts	
Wu et al 2019[153]	CRISPR-Cas9	Double guide	Deletion of part of exon 1 from just upstream of start codon to downstream of the CAG repeat	HEK293 cells	
Lopes et al 2020[154]	CRISPR-Cas9	Double guide	Deletion of part of exon 1 from just upstream of start codon to exon1-intron1 boundary	HD patient-derived iPSCs	

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the pre-mRNA and is therefore quite upstream in the molecular pathology. However, this approach is not specific for the mutant allele and, as it targets a downstream exon, is also not expected to affect HTT-ex1 production or toxic RNA gain-of-function.

In a more indirect fashion, metformin has been shown to reduce translation of HTT through interacting with the MID1/PP2A/mTOR protein complex.[158] Interestingly, the effect of metformin was found to be specific for mHTT and to also impact HTT-ex1 protein formation. The drug can be administered orally, and as it is already in clinical use for the treatment of diabetes, its safety profile has already been well established. Metformin is currently being tested for the treatment of HD in a phase III clinical trial to establish its potential as a treatment for HD (NCT04826692). Although it has been shown to reduce HTT levels, RNA-mediated toxicity is not expected to be targeted by its mechanism of action.

There are no therapies that target HTT-ex1 exclusively, but some therapies target HTT-ex1 in addition to the full-length HTT. The most advanced is uniQure's gene therapy AMT-130, which is currently being tested in phase I/II clinical trials (NCT04120493 and NCT05243017). AMT-130 is an AAV5-delivered miRNA which is administered through a one-time intrastriatal injection. This therapy is not allele-selective, and its effect on RNA-mediated toxicity has not yet been established.

Several other HTT-ex1 targeting candidates are close to entering clinical trials, including Galyan Bio's HTT-ex1 binding small molecule GLYN122 and Vybion's INT41 intrabody. These therapeutic candidates target the protein and are therefore not expected to impact RNA-mediated toxicity. According to the companies' websites, both are performing IND-enabling studies, although their target date to enter the clinic is not clear (https://www.galyan.bio/pipeline, https://www.vybion.com/?page=product_pipeline).

Likewise, Vico Therapeutics received FDA orphan drug designation for their CAG-targeting ASO in July 2021 and is expected to start clinical trials soon (https://vicotx.com/pipeline/). Takeda and Sangamo are further developing their ZFP approach targeting the CAG repeat (https://www.sangamo.com/programs/). Both approaches preferentially target mHTT and as they act on the (pre-)mRNA and on transcription, respectively, these drug candidates may also have a beneficial effect on RNA-mediated toxicity.

Discussion

Although all the approaches mentioned, as well as others in earlier phases of development, aim to reduce HTT levels, their mechanism of action is different and not all pathways related to HTT toxicity will be engaged. The relative contribution of each pathway is a matter of debate and is likely to depend on many factors, including age, tissue and cell type. Several of the described mechanisms of N-terminal HTT fragment production, including calpain cleavage and premature polyadenylation, have been shown to correlate with repeat length. This is also the case with *HTT*-ex1 formation through aberrant splicing. Therefore, it may be expected that as the repeat gets longer over time due to somatic instability, the contribution of these mechanisms will increase. Nonetheless, the broad molecular pathology of HD would likely benefit most from an intervention that acts as far upstream as possible, ie, on the DNA or the RNA level.

For an approach to be successful in disease modification, next to efficiency, adequate safety is key. Safety issues can arise from intrinsic characteristics of the therapeutic modality itself (eg, chemistry, properties of the therapeutic vector, and need of chronic administration), which are not covered in this review. The mechanism of action of the approach can also have different safety risks. Very specific approaches, with a well-understood mechanism, and with low to no interactions with other processes and molecules other than those related to HTT toxicity, would be preferred.

Multiple different approaches are running head-to-head. The small molecule splicing modulators are among the most elegant in terms of delivery, as these are capable of crossing the blood–brain barrier and can therefore be administered orally. However, these small molecules are not specific for mHTT or even solely for HTT, and long-term studies are needed to determine the safety profile. Furthermore, these splicing modulators are expected to affect neither aberrant splicing of HTT-ex1 nor toxic RNA gain-of-function effects. ASOs and siRNAs have a less favorable distribution and need to be administered locally, although novel chemistries, such as peptide nucleic acids and di-siRNAs, have shown more promising biodistribution and may allow for systemic administration. These synthetic oligonucleotides are active for a limited amount of time, and therefore need to be readministered frequently. CAG-targeting ASOs are expected to not only reduce HTT and HTT-ex1 protein gain-of-function but also to alleviate RNA-mediated toxicity; however, non-specific effects on other genes containing CAG repeats may be difficult to overcome. Finally, the gene therapy approaches utilize AAVs to deliver their cargo. The

current generation of AAVs is not sufficiently capable of crossing the blood–brain barrier and therefore still needs to be administered locally, although efforts are ongoing to identify novel capsids that could be administered in a less invasive manner, eg, Goertsen et al.[159] Because most cells that are targeted in HD are non-dividing, a more invasive route of administration is, however, less of an issue, as the therapy would only need to be administered once. uniQure's miRNA-based strategy would reduce toxic protein gain-of-function, whereas Takeda and Sangamo's ZFP approach targets DNA and thereby acts upstream of mHTT transcription, which would improve both toxic protein- and RNA gain-of-function; yet, as the mechanism of action of this approach involves direct targeting of the repeat, off-target effects may be an issue. Pre-clinically, gene editing approaches using CRISPR-Cas are being explored. However, long-term studies will need to show the safety profiles of such approaches.

To maximize therapeutic efficacy, future research will need to point out whether it may be advantageous to combine various therapeutic strategies with different modes of action. Further, it is likely that any therapeutic approach will benefit from as early intervention as possible. To this end, excellent safety profiles and good biomarkers of both safety and efficacy will be key.[160]

Conclusion

In summary, we have reviewed the production of N-terminal HTT protein fragments, their role in HD pathology, as well as therapeutic approaches to target these toxic species. Extensive research into HD continues to deepen our understanding of the broad molecular mechanisms leading to disease. With the increasing understanding of the pathological mechanisms associated with mHTT, several different therapeutic approaches are being developed, which will hopefully lead, in the near future, to halting or modification of this devastating disease.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

LB, ME and AV are employees of, and may own stock/options in, uniQure biopharma B.V. In addition, Dr Astrid Vallès has a patent WO2021053018 issued to UNIQURE IP B.V. The authors report no other conflicts of interest in this work.

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