Targeting for success: mechanistic insights into microRNA-based gene therapy for Huntington disease

Sogorb Gonzalez, M.

Chapter 1

Targeting for success: Mechanistic insights into HTT-lowering therapies for Huntington disease

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Abstract

Gene therapy is emerging as a potential treatment for untreatable neurodegenerative diseases. Huntington Disease (HD) is the most common inherited neurogenerative disease caused by a trinucleotide repeat expansion in the huntingtin (HTT) gene, giving rise to a toxic mutant HTT (mHTT) protein. The well-defined monogenic cause makes HD a good target for gene therapy approaches and several novel therapeutics are being developed with the aim of reducing the production of the mHTT. In view of recent failures of some therapeutics to translate into patient benefit, it is essential to precisely understand the pathogenic mechanisms that are currently targeted as well as the mechanism of action of the HTT-lowering therapies in development. We here review our current understanding of the molecular pathology of HD, how to specifically target the critical pathogenic mechanisms and how to determine therapeutic efficacy. Finally, we discuss the current challenges for HTT-lowering therapies and the ongoing advances of gene therapy treatments to overcome these therapeutic limitations.

Introduction

Huntington Disease (HD) is an ultimately lethal, genetic neurodegenerative disease that typically manifests in adulthood with motor, cognitive and psychological symptoms. Symptomatic onset is characterized by the appearance of chorea and muscle rigidity, often preceded or accompanied by neuropsychiatric symptoms like depressed mood, mania, irritability and psychosis (Ross *et al.*, 2014). HD is dominantly inherited and is the most common genetic disease affecting the central nervous system (CNS) with a prevalence of 1 to 9 in 100.000 people, depending on the ethnicity and population (Rawlins *et al.*, 2016). Symptomatic therapies are used to treat motor and psychological symptoms and may have a temporary beneficial effect on motor function and quality of life (Bachoud-Lévi *et al.*, 2019). Unfortunately, despite decades of research and ongoing clinical trials, there is still no established treatment to attenuate the natural course of this devastating disease.

HD is caused by a trinucleotide CAG repeat expansion in exon 1 of the huntingtin (*HTT*) gene (MacDonald *et al.*, 1993). Individuals who inherit ≥ 40 CAG repeats will develop HD given a normal lifespan, with longer repeats resulting in earlier age of onset. Expansions with 36-39 CAG repeats are considered alleles with reduced penetrance. The expanded CAG repeat sequence results in the transcription of a mutant form of huntingtin protein (mHTT) containing an expanded polyglutamine (polyQ) tract in the N-terminal domain. This long polyglutamine tract confers a toxic gain-of-function to the mHTT protein which has been associated with neuronal dysfunction mechanisms including transcriptional dysregulation, proteasome overload, excitotoxicity, mitochondria and synaptic dysfunction, and eventually, cell death (Jimenez-Sanchez *et al.*, 2017; Tabrizi *et al.*, 2020).

The fact that mHTT is expressed since birth and in all tissues, but only becomes pathological in adulthood and initially in striatal neurons, raises the question whether mHTT, or other potentially related mechanisms, are the main driver of HD pathology (Jimenez-Sanchez *et al.*, 2017). Moreover, the CAG length-dependent age of onset also suggests that the relationship between mHTT protein and HD pathogenesis might be more complex. These considerations have become relevant in view of the recent finding that therapies exclusively reducing the mHTT protein have not achieved the expected therapeutic benefits (Kingwell, 2021).

Hence, understanding the different mechanistic aspects of HD pathology and disease-modifying approaches will forward the development of successful therapies. In this chapter, we introduce the current knowledge in the field, as well as therapeutic limitations related to HD pathology, drug modalities and measurements of efficacy, with special focus on HTT-lowering therapies. For this, the field has been categorized into the following questions (**Figure 1**):

- ***WHAT?***: Which pathological mechanisms contribute to HD and what is the most promising target to stop neurodegeneration and HD progression?
- ***HOW?***: Which currently available technologies are the most suitable to target these mechanisms?
- ***WHERE?***: Which brain regions do we need to target to achieve significant therapeutic effect and which technologies can accomplish this?
- ***HOW GOOD?***: Which models and outcomes are used to measure efficacy for successful translation to patients? Which new markers should be developed for gene therapy studies?
- ***HOW BAD?***: What are the toxicities and immunogenic responses associated with the treatment?

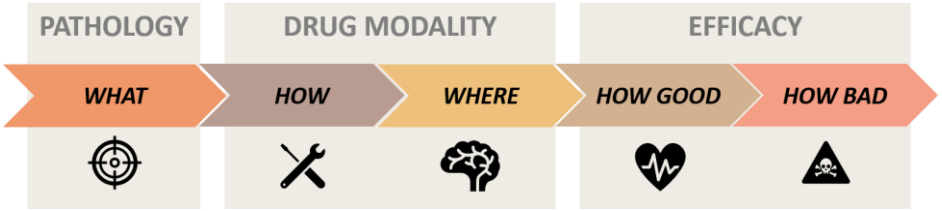

Figure 1: Important questions to investigate the disease pathology and potential targets, properties of current drug modalities, and outcomes of efficacy and safety with the goal to develop successful therapies for HD and other neurodegenerative diseases.

WHAT? – Pathological molecular mechanisms in HD

Despite its monogenic cause, HD pathology is not fully understood and curative therapies are not yet available. A better understanding of the pathological mechanisms leading to the gradual loss of striatal neurons and the midlife onset of symptoms will enable the development of novel therapeutic approaches.

Initially, it was generally accepted that the expression and aggregation of mHTT was the direct toxic molecular driver of HD pathogenesis and early studies in post-mortem patients identified mHTT inclusions in the striatum and cortex as the pathological hallmark of HD (DiFiglia, 1997). At the same time, the first genetic mouse models demonstrated that the incorporation of a long CAG expansion in *HTT* homologous mouse gene or the expression of mHTT cDNA sequences also led to mHTT inclusions, neuronal death and HD-like phonotype in mice (Mangiarini *et al.*, 1996; Menalled *et al.*, 2002). These studies supported the toxic gain-of function of mHTT and the hypothesis that the prevention of mHTT aggregate formation should be a promising therapeutic strategy for treatment of HD. During the last therapies (Wild and Tabrizi, 2017, Tabrizi *et al.*, 2019*a*; Marxreiter *et al.*,

2020). However, the lack of efficacy of some of such approaches raised concerns about the notion that mHTT constitutes a valid therapeutic target (Kingwell, 2021).

The CAG repeat expansion does have consequences for neuronal biology that extend beyond the production of mHTT aggregates (Tabrizi *et al.*, 2020; Heinz *et al.*, 2021). These include somatic DNA repeat instability, formation of aberrant spliced toxic transcripts and nuclear RNA foci, sequestration of proteins involved in transcriptional regulation and impairment of proteasome function, among others. Recent research has investigated the contribution of these processes as potential drivers of the striatum-selective and age-dependent pathogenesis.

The expanded CAG repeat is unstable and undergoes a progressive increase in length throughout patient's life, process known as somatic instability (Swami *et al.*, 2009). Somatic instability occurs in a tissue-specific manner in HD patients, with striatal neurons undergoing a dramatic mutation length increase up to >1000 CAG repeats (Kennedy *et al.*, 2003). A novel mouse model of somatic instability with uninterrupted CAG repeats (BAC-CAG model) showed significant correlations between somatic instability in the striatum and nuclear mHTT aggregation with the onset of behavioral impairments and other molecular phenotypes (transcriptomic dysregulation and reactive gliosis) which closely resemble HD clinical pathology (Gu *et al.*, 2022). In addition, GWAS studies identified genes involved in DNA mismatch repair (MMR) as contributors of somatic instability disease and worse HD outcomes in HD patients (Lee *et al.*, 2015; Ciosi *et al.*, 2019; Roy *et al.*, 2021). Altogether, these studies support the hypothesis that somatic instability, leading to CAG repeat expansions greatly exceeding the germ-line number, contributes to the onset and progression of striatum-selective pathology in HD.

Not all mHTT protein species are equally pathogenic, and increased somatic CAG expansion in striatal neurons is considered to contribute to the production of the highly toxic exon 1 HTT (HTTex1) fragment through aberrant splicing. Mutant *HTT* pre-mRNA undergoes incomplete splicing of exon1 to exon 2 resulting in the production of a short HTTex1 protein (Sathasivam *et al.*, 2013). Furthermore, in an HD patient's tissue, this mis-spliced HTT transcript can be detected (Neueder *et al.*, 2017). The severe toxicity induced by HTTex1 has been demonstrated in different HD animal models and indeed the fastest progressing HD mouse model is based on the overexpression of HTTex1 fragment (Mangiarini *et al.*, 1996; Barbaro *et al.*, 2015). Phenotype onset correlates with levels of HTTex1 splicing in mouse models with similar CAG repeats (Franich *et al.*, 2019). Importantly, the suppression of HTTex1 expression in a conditional model was able to reverse aggregate formation and motor decline (Yamamoto *et al.*, 2000), suggesting that HTTex1 reduction is beneficial for therapeutic efficacy in HD. Longer CAG repeats correlate with increased aberrant splicing and HTTex1 levels (Neueder *et al.*, 2018) and therefore

toxic HTTex1 formation is expected to occur first in neurons that display somatic expansions, such as striatal neurons (Kennedy *et al.*, 2003). For these reasons, it is currently thought that lowering levels of HTTex1 protein will have a greater therapeutic benefit than exclusively targeting the full-length mHTT. Unfortunately, most of the current therapies in preclinical and clinical studies are based on genetic approaches that target sequences downstream exon 1 and therefore are not expected to reduce the translation of the HTTex1 protein.

In conclusion, novel molecular findings, such us somatic instability, CAG repeat expansions and increased generation of toxic HTTex1 fragments, open the door to new therapeutic approaches in HD. In particular, HD pathogenesis is now thought to be a two-step event (presumably including CAG length expansions and HTTex1 production) rather than a result of direct full-length mHTT-induced toxicity (Neueder *et al.*, 2017). According to this model, somatic CAG repeat instability that first accumulates in striatal neurons during the patient's pre-symptomatic years, and not the congenital number of CAG repeats, would trigger the formation of toxic drivers, such as HTTex1, up to a cell type-specific lethal threshold that results in the neurodegeneration of vulnerable cells and disease manifestation(Neueder *et al.*, 2017; Pinto *et al.*, 2020) **(Figure 2).** Consequently, therapies that stop somatic instability or reduce the levels of toxic HTTex1 fragments, ideally in striatal neurons in early disease state, may be potential treatments for HD patients.

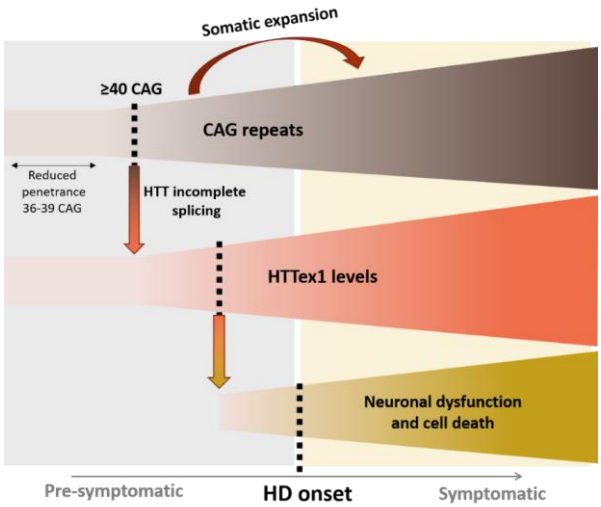

Figure 2: Two-step model of HD pathogenesis. Based on this model CAG expansions, which increase via somatic instability (step 1), induce and accelerate the formation of the pathogenic HTTex1 fragment by aberrant splicing (step 2) up to a threshold level in which induces neuronal dysfunction and cell death during pre-symptomatic phase. Dotted lines represent the "threshold level" that induces the next process.

HOW? - HTT-targeting therapeutic modalities

As a consequence of a better understanding of HD pathology, current therapeutic approaches have shifted from symptomatic to molecular disease-modifying treatments. With the aim to reduce mHTT-induced toxicity, molecular treatments have been used to edit, silence or reduce mHTT expression and protein translation (Tabrizi *et al.*, 2019*a*; Leavitt *et al.*, 2020; Marxreiter *et al.*, 2020). The efficacy of each of this HTT-lowering approaches would mainly depend on where the intervention targets the HTT pathway: DNA, RNA or protein (Wild and Tabrizi, 2017). The most advanced HTT-targeting therapies are illustrated in **Table 1**.

- DNA-targeting treatments: DNA-targeting therapeutics would be ideal to address all aspects of CAG-induced toxicities. Although challenging, these technologies aim at either removing the mutated gene or inhibiting the transcription. The two technologies in development include zinc finger protein (ZFP) and the novel CRISPR-Cas9 (Yang *et al.*, 2017; Zeitler *et al.*, 2019). Both approaches are gene therapy products mediated by the expression of therapeutic proteins delivered by viral vectors.

- RNA-targeting treatments: RNA-targeting approaches are the only genetic therapies that have been tested in clinical trials to date. These treatments aim at inducing *mHTT* mRNA degradation, consequently resulting in reduction of mHTT protein and aggregates. Three molecular therapeutics have been used: antisense oligonucleotides (ASO) (Carroll *et al.*, 2011; Kordasiewicz *et al.*, 2012, Tabrizi *et al.*, 2019*a*), RNAi interference molecules (microRNA (miRNA), small interfering RNA (siRNA) and short hairpin RNA (shRNA)) (Rodriguez-Lebron *et al.*, 2005; McBride *et al.*, 2011; Miniarikova *et al.*, 2016; Alterman *et al.*, 2019), and small molecules (Bhattacharyya *et al.*, 2021). The various approaches importantly differ in their therapeutic efficacy, CNS target area coverage and durability. Importantly, and often overlooked, is the fact that the mRNA target sequence critically determines the lowering of aberrantly-spliced mHTT transcripts, such as HTTex1 (Sathasivam *et al.*, 2013). Therapies targeting sequences downstream of exon 1 will generally not affect the production of HTTex1, despite lowering the full-length mHTT protein.

- Protein-targeting modalities: These include the direct targeting of mHTT with intracellular antibodies (Southwell *et al.*, 2009), the modulation of degradation pathways such as autophagy and proteasome (Soares *et al.*, 2019), and the inhibition of proteolytic cleavage to reduce toxic N-terminal fragments (except for HTTex1 fragment) (Wellington *et al.*, 2000).

Table 1: Most advanced HTT-targeting therapies for HD.

Sponsor	Drug name	Clinical status	WHAT? (Target)	HOW?	WHERE? (injection route)	Reference
ASO & siRNA						
Ionis/Roche	Tominersen /RO7234292	Phase 1b/2 Phase 3 – *Suspended – lack of efficacy*	RNA (Target: *HTT* exon 36)	ASO	Intrathecal	(Kordasiewicz *et al.*, 2012, Tabrizi *et al.*, 2019) **NCT03761849**
Wave Life Sciences	WVE-120101 WVE-120102	Phase 1b/2a- *Suspended – lack of efficacy*	RNA (Target: mHTT SNP)	ASO	Intrathecal	**NCT04617847** **NCT04617860**
	WVE-003	Phase 1b-2a- recruiting	RNA (Target: mHTT SNP)	ASO	Intrathecal	**NCT05032196**
Alnylam	ALN-HTT	Preclinical	RNA (Target: *HTT* exon 1)	siRNA (cholesterol-conjugated)	Intraparenchymal	(DiFiglia *et al.*, 2007)
Atalanta		Preclinical	RNA (Target: 3' UTR)	siRNA (divalent-siRNA)	Intrastriatal	(Alterman *et al.*, 2019)
Biomarin/Vico		Preclinical	RNA (Target: CAG repeat)	ASO		(Datson *et al.*, 2017)
Nanjing University, China		Preclinical	RNA (Target: *HTT* exon 1)	Exosome-mediated siRNA	Intravenous	(Wu *et al.*, 2018)

Continuation Table 1

Sponsor	Drug name	Clinical status	WHAT? (Target)	HOW?	WHERE? (injection route)	Reference
Gene therapy approaches (ZFP, CRISPR and miRNA)						
Takeda/ Sangamo	TAK-686	Preclinical	DNA – CAG repeat	AAV-ZFP	Intraparenchymal	(Zeitler *et al.*, 2019)
Imperial college London		Preclinical	DNA – CAG repeat	AAV-ZFP	Intraparenchymal (striatum)	(Garriga-Canut *et al.*, 2012)
Emory university		Preclinical	DNA – CAG repeat	AAV-CRISPR/Cas9	Intraparenchymal	(Yang *et al.*, 2017)
uniQure	AMT-130 (AAV-miHTT)	Phase 1b/2 - *Ongoing*	RNA (Target: *HTT* exon 1)	AAV5-miRNA (pre-miR-451)	Intraparenchymal (striatum)	(Miniarikova *et al.*, 2016) **NCT04120493**
Voyager	VY-HTT01	Phase 1b – *Recruiting*	RNA (Target: *HTT* exon 2)	AAV1-miRNA (pre-miR-30)	Intraparenchymal Putamen-thalamus	(Stanek *et al.*, 2014) **NCT04885114**
Spark		*Preclinical*	RNA (Target: *HTT* exon 52)	AAV1-miRNA (pre-miR-30)	Intraparenchymal: Putamen	(McBride *et al.*, 2011)
University of Massachusetts		Preclinical	RNA (Target: *HTT* exon 48)	AAV9-miRNA (pre-miR-155)	Intraparenchymal (Striatum and cortex)	(Pfister *et al.*, 2017)
Small molecules						
PTC therapeutics	PTC518	Preclinical	RNA - Splicing modulator	Small molecule	Oral	Bhattacharyya et al. abstract at HSG 2021
Novartis	Branaplam	Phase 1	RNA – Splicing modulator	Small molecule	Oral	(Keller *et al.*, 2022)
Others						
Triplet therapeutics	TTX-3360	Preclinical/IND	*MSH3* gene (DNA damage repair)	ASO	Intracerebroventricular (ICV)	Antonijevic et al. presentation at EHDN 2021 meeting

Gene therapy for HD

Gene therapy was proposed as a therapeutic tool 30 years ago to deliver genetic material to cells with the aim to alter gene expression (Friedmann, 1992). The DNA material is delivered using a carrier (vector), most commonly based on an adeno-associated virus (AAV), and is then transcribed utilizing the cell's own machinery resulting in continuous expression of the therapeutic transgene. Depending on the nature of the transgene, gene therapy can be used to correct defective genes by introducing a functional copy, to silence mutant alleles using RNAi or to deliver gene-editing technologies (Piguet *et al.*, 2017; Papanikolaou and Bosio, 2021). In HD, the molecular therapeutics tested in a gene therapy modality include the expression of HTT-targeting miRNAs and shRNAs (Rodriguez-Lebron *et al.*, 2005; Miniarikova *et al.*, 2016), CAG-targeting ZFP and CRISPR-Cas9 molecules (Yang *et al.*, 2017; Zeitler *et al.*, 2019), and mHTT-targeting intrabodies (Southwell *et al.*, 2009). The main advantage of gene therapy for brain diseases is that a single administration in affected brain areas, although invasive, can potentially result in long-term correction of disease pathology and lifelong treatment.

WHERE? - Therapeutic coverage of affected brain areas in HD

A basic principle in drug development is that the therapeutic drug should distribute to the diseased target tissues. HD pathology is characterized by intranuclear and cytoplasmic insoluble aggregates of mHTT in neuronal cells (DiFiglia, 1997), and medium spiny neurons (MSN) in the striatum are the primary affected neurons in early-stage HD. Indeed the characteristic pathology in HD patients comprise striatal atrophy and enlargement of the lateral ventricles (Hobbs *et al.*, 2010). As the disease progresses, the loss of neurons extends to other areas, including the deep layer cortical neurons and substantia nigra (Rosas *et al.*, 2006; Tabrizi *et al.*, 2009) and by end stage, typically more than 30% of the brain mass is lost (De La Monte *et al.*, 1988). Therefore, therapeutics targeting HD molecular disease-causing mechanisms, need to effectively distribute to the striatum, and secondarily to other brain areas, in order to achieve a fully effective outcome (Wang *et al.*, 2014). For CNS diseases, the complex brain anatomy and the blood brain barrier (BBB) protection of the brain result in a limited drug distribution which is considered one of the major challenges in the development of treatments for brain diseases. It also has become apparent that results obtained in small animal models often do not translate well to large animals and humans (Eaton and Wishart, 2017).

Three different routes of administration can be used to delivery therapeutics in the brain: intravenous, intrathecal and intraparenchymal (Hocquemiller *et al.*, 2016). Currently,

only the last ensures sufficient delivery of DNA oligonucleotides, siRNAs or gene therapy vectors to deep brain structures such as the striatum. Stereotactic intraparenchymal administration is invasive and cumbersome and the approach would only be suitable for single-dosed approaches with long-term effects such as gene therapeutics (Samaranch *et al.*, 2017). The delivery of various mHTT-lowering RNAi strategies into the striatum, or cerebral ventricles was associated with the reduction of mHTT aggregation and behavioral improvement in HD animal models (Rodriguez-Lebron *et al.*, 2005; DiFiglia *et al.*, 2007; Stanek *et al.*, 2014; Miniarikova *et al.*, 2017; Didiot *et al.*, 2018; Spronck *et al.*, 2019).

The toxicity of misfolded pathogenic proteins and fragments can spread throughout the brain by seeding protein aggregation in recipient cells. Spreading of mHTT between cells is evident from in vitro and animal experiments showing that mHTT aggregate transmission between neurons contributes to HD pathology (Pecho-Vrieseling *et al.*, 2014; Jeon *et al.*, 2016; Ananbeh *et al.*, 2021). One of the mechanisms of intercellular transfer is mediated by the secretion and dissemination of mHTT within extracellular vesicles (EV) (Jeon *et al.*, 2016). EVs are a heterogenous group of nanovesicles, including exosomes and microvesicles, that contain important biological cargos such as cellular miRNAs, long non-coding RNAs, proteins, lipids and DNA (Valadi *et al.*, 2007). Molecular therapies might be able to distribute between affected neuronal cells in the same manner as pathological proteins such as mHTT contributing to improve therapeutic efficacy as disease advances.

HOW GOOD and HOW BAD? – Measuring efficacy and safety of HTT-lowering treatments

In order to assess the therapeutic success of potential treatments it is important to establish a panel of outcomes for both positive and negative effects that are critical for disease progression. Outcomes of efficacy ("how good") include measurements of target engagement, functional improvement and translational biomarkers predictive of the disease advancement (**Figure 3**). For HTT-lowering therapies, on-target lowering efficacy has been assessed in preclinical studies by measuring the reduction of FL-mHTT protein levels and mHTT aggregates within affected CNS regions. For this, numerous in vitro cultures and animal models have been used, including large transgenic models such as HD transgenic minipigs (Baxa *et al.*, 2013; Howland and Munoz-Sanjuan, 2014; Miniarikova *et al.*, 2018). Since brain biopsies are too invasive, it is not feasible to measure lowering efficacy directly in patients' brain. The levels of mHTT (FL-mHTT) in cerebral spinal fluid (CSF) were proposed as markers of on-target efficacy in patients' CNS and have been used in the first clinical trials (Tabrizi *et al.*, 2019). However, it is not clear to which extend the concentrations of mHTT in the CSF reflect the concentrations of mHTT in deep brain areas, or instead and most likely,

they reflect concentrations in spinal cord and cortex, according to the unsuccessful clinical trial with intrathecal infusion of ASOs (Tabrizi *et al.*, 2019). Another biofluid markers commonly used in brain diseases is the concentrations of neurofilament light chain (NfL), a general marker of neuroaxonal damage, in the CSF (Byrne *et al.*, 2018). NfL levels is a good predictor of disease onset, but does not correlated with symptom progression after symptom onset (Byrne *et al.*, 2018; Parkin *et al.*, 2021). Therefore, it is not clear whether it could be a suitable biomarker for response to neuroprotective treatments (Tabrizi *et al.*, 2019). It is important to mention, that although ideal due to their recent contribution in HD pathogenesis, measurements of HTTex1 production or somatic instability in biofluids have not been assessed yet. Measuring these events is challenging since levels are low and first changes mainly take place in striatal neurons.

Other studied biomarkers for early prediction of therapeutic outcomes include volumetric measures by structural magnetic resonance imaging (MRI) (Wilson *et al.*, 2018). In HD patients, striatal volume was identified as the best variable to track longitudinal progression (Abeyasinghe *et al.*, 2021). Hence, monitoring volumetric changes may provide a more reliable measurement of therapeutic improvement. Moreover, imaging motor tracts in thalamus and striatum, first susceptible areas to degeneration, may help to determine timing of treatment and efficacy in pre-manifest patients (Rosas *et al.*, 2006; Zeun *et al.*, 2022).

Safety is a major aspect of drug development, especially for gene targeting therapies which can induce unwanted off-target downregulation of other genes (**Figure 3**). For vector-based gene therapeutics, due to their persistent and irreversible nature, evaluation of long-term HTT-lowering effects as well as potential off-target effects is important (Murlidharan *et al.*, 2014; Keskin *et al.*, 2019). Moreover, pre-existing neutralizing antibodies and AAV-induced immune responses may contribute to treatment durability and patient safety. Most HTT-lowering therapies are non-allele-selective and the potential toxicity of long-term wild-type HTT (wtHTT) lowering is a major safety concern (Kaemmerer and Grondin, 2019). A whole set of preclinical studies, together with first clinical trial, indicates that partial lowering of wtHTT up to a certain extend is well-tolerated for at least 7 months in humanized HD mice (Caron *et al.*, 2020), 6 months in NHP (Grondin *et al.*, 2012) and 4 months in HD patients (Tabrizi *et al.*, 2019)

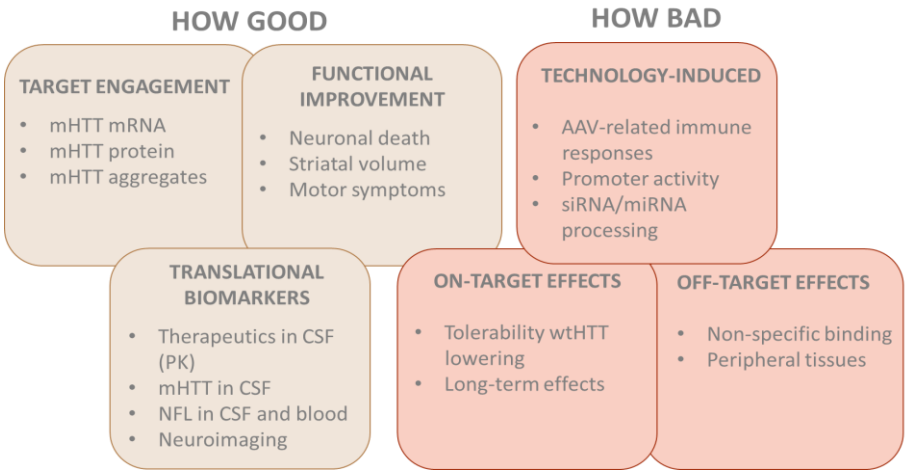

Figure 3: **Measurements of efficacy ("how good") and safety ("how bad") for HTT-lowering therapies.**

Conclusion

During the last decades, great efforts have led to the development and clinical testing of potential therapeutics for the treatment of HD. However, we are not there yet. Altogether, this mechanistic overview of molecular drivers of HD pathology, current therapeutic modalities and outcomes of efficacy illustrates critical requirements and remaining challenges to achieve therapeutic success. Potentially therapeutic approaches should aim to target the most toxic species in the most affected brain areas. For instance, the reduction of HTTex1 fragments or the suppression of somatic instability, rather than the lowering of full-length mHTT protein alone, are suggested as superior targets to effectively stop disease onset and progression. Next, therapeutic targeting should be preferentially and evenly achieved in earlier and most affected brain areas (i.e striatum) and cells, and secondly in other brain areas in line with disease progression. Finally, development of sensitive indicators of early disease changes are needed to correctly assess these therapies in slow progressing HD patients.

Scope of the thesis

Although HD has a well-defined monogenic cause and promising HTT-lowering therapies are being tested in clinical trials, mechanism of action studies can reveal relevant information about effective targets and outcomes required for successful translation into patients. One of the most advanced HTT lowering therapies for HD is a micro(mi)RNA-based gene therapy which consists of an engineered miRNA targeting the exon 1 sequence of HTT (miHTT) and delivered by adeno-associated virus (AAV) into neuronal striatal cells (AAV-miHTT). AAV-miHTT treatment has previously demonstrated efficacy and safety in reducing mutant HTT protein and rescuing HD phenotype in several HD murine models (Miniarikova *et al.*, 2016, 2017; Spronck *et al.*, 2019; Caron *et al.*, 2020), in transgenic minipigs (Evers *et al.*, 2018) and in HD patient-derived cells (Keskin *et al.*, 2019). However, mechanism of action studies are still limited.

The work in this thesis describes novel mechanistic features of AAV-miHTT treatment for HD, including the targeting of different HTT species, the therapeutic spread between neuronal cells and the development of translational biomarkers to monitor its effect in the affected brain regions.

In **Chapter 2** describes the reduction of highly toxic exon 1 HTT fragments, as well as full-length mutant HTT, in the brain of HD mouse models by AAV-miHTT.

Chapter 3 describes the widespread and sustained lowering efficacy of AAV-miHTT in disease-relevant regions in a large brain in HD transgenic minipigs, and the potential of using biofluids markers to determine vector expression and efficacy in the clinic.

Chapter 4 and **5** describe a novel mechanism of secretion and dissemination of engineered miRNAs mediated by extracellular vesicles (EV). Circulating engineered miRNAs in biofluids were used as sources of pharmacokinetic markers to monitor durability of miRNA therapeutics in the brain of non-human primates. Moreover, the uptake of EV-enriched engineered miRNAs by neighboring cells resulted in gene silencing in recipient cells, indicating therapeutic spread beyond AAV transduction.

Chapter 6 provide a general discussion of the main findings on the thesis and its implications for gene therapies for HD and other neurodegenerative diseases.

In the light of the work of this thesis, we support the reduction of HTTex1 fragment, the persistent efficacy in most affected brain areas, and mechanisms that improve therapeutic spread to all affected cells, as processes that potentially contribute to the successful treatment of HD patients.

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