MicroRNA-based gene therapy for Huntington's disease : Silencing the villain

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GENERAL INTRODUCTION

Translation of microRNA-based huntingtin lowering therapies from preclinical studies to the clinic

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ABSTRACT

The single mutation underlying the fatal neuropathology of Huntington's disease (HD) is a CAG triplet expansion in exon 1 of the huntingtin (*HTT*) gene, given rise to a toxic mutant HTT protein. There has been a number of not yet successful therapeutic advances in the treatment of HD. The current excitement in the HD field is due to the recent development of therapies targeting the culprit of HD either at the DNA or RNA level to reduce the overall mutant HTT protein. In this review, we briefly describe short-term and long-term HTT lowering strategies targeting HTT transcripts. One of the most advanced HTT lowering strategies is a micro (mi)RNA-based gene therapy delivered by a single administration of an adeno-associated viral (AAV) vector to the HD patient. We outline the outcome measures for the miRNA-based HTT lowering therapy in the context of preclinical evaluation in HD animal and cell models. We highlight the strengths and ongoing queries of the HTT lowering gene therapy as an HD intervention with a potential disease-modifying effect. This review provides a perspective on the fast-developing HTT lowering therapies for HD and their translation to the clinic based on the existing knowledge in the preclinical models.

INTRODUCTION

Huntington's disease (HD) is the most common autosomal dominant neurodegenerative disorder with the prevalence rate 1-10 in 100.000 individuals worldwide.[1,2] The genetic cause of HD is an expansion of more than 39 CAG triplets in exon 1 of the huntingtin (*HTT)* gene, which results in a toxic gain of function of the mutant HTT protein containing a long polyglutamine (polyQ) tract.[3] Carriers of 36 to 39 CAG repeats show reduced penetrance.[3,4] Mutant HTT protein causes neuropathology affecting the entire brain, with medium spiny neurons of the striatum being particularly vulnerable at early stages.[5,6] The clinical symptoms include progressive motor, cognitive, and psychiatric disturbances.[5,7] The length of HD expansion on average consists of 40-55 CAGs and roughly predicts the motor onset in an inverse manner, with a 38-56% variance introduced by yet unidentified genetic modifiers.[8] The age of motor onset is typically in mid-life with a median survival of 15-18 years.[7] Occasionally, HD manifests in juveniles with a typical severe phenotype due to the very long CAG expansion.[9] Similar to other incurable neurodegenerative disorders, HD is devastating for patients and their families.

The mutant HTT protein mediates toxicity by intervening in crucial cellular pathways such as apoptosis, protein degradation, transcription, axonal transport, and mitochondrial function, leading to cellular dysfunction and cell death.[6,10] A consistent key feature of HD pathogenesis is the aggregation of mutant HTT protein in different conformations and at various cellular localizations.[11–13] Although the exact role of HTT aggregation is still under debate, several findings indicate that HTT aggregates are directly linked to HD pathology. Overexpressing an aggregating N-terminal fragment of mutant HTT is sufficient to cause HD-like neuropathology in transgenic HD mice and in a lentiviral HD rat model,[14,15] and blocking polyQ-mediated aggregation has neuroprotective effects in transgenic HD mice.[16] Interestingly, the mutant HTT aggregates can be transmitted by a prion-like mechanism to genetically unrelated fetal neural allografts within a brain of HD patients, suggesting a propagation of pathology that is similar to other neurodegenerative disorders such Alzheimer's and Parkinson's disease.[17–19] Apart from the HTT aggregation, a formation of mutant HTT fragments of different lengths has been implicated as an essential event in HD pathology.[20] The generation of these fragments is not fully understood. The mutant HTT protein undergoes a proteolytic cleavage resulting in N-terminal HTT fragments that showed toxicity in transgenic mice and have been found in HD human brains *post mortem*.[21–24] Short HTT exon 1 fragments can be translated from an aberrant splice variants in knock-in HD mice and these fragments have been also localized in the brain of HD patients *post mortem*.[25] Lastly, similar to other repeat-associated disorders, mutant HTT RNA showed repeat-associated non-ATG (RAN) translation that generates short HTT fragments of which some are toxic.[26]

Early therapeutic HD strategies focused on a development of agents to counter the downstream toxic cellular effects of mutant HTT protein or on cellular replacement strategies to compensate for the loss of neurons.[27] Unfortunately, this has not yet resulted in an effective HD treatment that would halt or delay disease progression. Because the mutant HTT protein seems to be the cardinal toxic trigger for the induction of HD pathogenesis, the HTT lowering is currently considered to be the main therapeutic objective for HD.[28] Even after the onset of symptoms, a conditional blockage of HTT expression is sufficient to clear HTT aggregates resulting in HD-like behavioral improvements in mice, which suggests that HD pathogenesis can be reversed after the onset of motor dysfunction.[29] An effective therapeutic HTT lowering approach would require a long-term HTT suppression in wide areas of the brain, which can be achieved using gene therapy technologies.[28]

Most HD patients are heterozygous for the CAG expansion and therefore, HTT lowering strategies are being developed and assessed in a non-selective or allele-selective manner by targeting both alleles or preferentially the mutant HTT allele, respectively.[30] Mutant allele-specific therapeutic strategies aim to develop molecules exclusively recognizing the longer CAG tract or sequences containing specific isoforms of heterozygous single-nucleotide polymorphisms (SNPs) that are in a linkage disequilibrium with the CAG expansion.[31–33] These approaches are challenging, because potential target SNPs are scarce within the HD population and because it is difficult to get sufficient allele selectivity.[31,32,34,35] Therefore, a majority of the most advanced projects focus on the non-selective HTT lowering.[28] Multiple lines of investigations using HD rodent and nonhuman primates indicate that the non-selective HTT lowering is feasible, reverses HD neuropathology, and –within a certain therapeutic window- is well tolerated.[36–39] The first ongoing HTT lowering clinical trial (https://clinicaltrials.gov/: NCT02519036) was initiated in 2015 and was designed to lower both HTT alleles by using antisense oligonucleotides delivered intrathecally (IT) to early stage HD patients.

Two main modes of HTT silencing are currently developed: short-term and long-term HTT lowering. The short-term HTT lowering is based on periodical re-administration of therapeutics that lower HTT, whereas the long-term HTT lowering uses viral vectors as delivery vehicles of therapeutic expression cassettes to achieve continuous drug influx in a target tissue.[20,40,41] One of the most advanced long-term HTT lowering strategies is a micro (mi)RNA-based gene therapy that comprises a single administration of an adeno-associated viral (AAV) vector delivering an expression cassette of a therapeutic miRNA precursor.[37,39,42,43] These precursors are designed to activate the endogenous mRNA silencing machinery to reduce overall HTT translation in target cells.

As for any other potential HD treatment, an AAV-miRNA gene therapy approach needs to address a representative set of outcome measures that are clinically relevant and support the translation of the therapy to the clinical setting. A great number of HD animal models, ranging from fruit flies to higher species such as a minipig or a sheep, have been

generated to evaluate early and late stage preclinical therapeutic approaches for HD.[44–46] Unfortunately, due to the complex nature of the disease, there is not a single model that addresses all necessary treatment outcome measures required for the initiation of a clinical trial. Therefore, the preclinical development of a therapy for HD must include a combination of several *in vitro* and *in vivo* studies in various disease models. The therapeutic benefit observed in a combination of HD cell and animal models is critical to support the rational for further clinical development.

In this review, we briefly discuss short-term and long-term HTT lowering strategies with a focus on miRNA-based gene therapies. We characterize the available HD animal or cell systems that enable preclinical testing of the AAV-delivered-miRNA-based gene therapy. The outcome measures that support the transition from preclinical studies to the clinic are thoroughly discussed. Ultimately, we propose a preclinical framework that should facilitate the preclinical study design for the RNA interference (RNAi)-based and other HTT lowering strategies.

HTT lowering strategies

Artificial DNA or RNA molecules to achieve lowering of HTT translation as a potential therapy for HD have been broadly investigated.[20] Here below, we will discuss these HTT lowering therapies into more detail.

Short-term HTT lowering

A wide range of small DNA and RNA molecules demonstrated efficacious short-term HTT lowering in HD rodent models and nonhuman primates.[20] Antisense oligonucleotides and small interfering (si)RNAs are designed to bind to HTT transcripts to halt HTT translation using either RNase H- or RNAi-based cellular silencing mechanisms, respectively. The chemical characteristics and mode of action of these therapeutics were extensively reviewed elsewhere.[41,47–51] Antisense oligonucleotides can dose-dependently suppress HTT, delay formation of mutant HTT aggregates, and improve neuropathology, as well as behavioral function in rodent HD models.[52–55]

Although HTT is widely expressed throughout the brain, the neuronal damage is more prominent within the corticostriatal circuitry involving cortex and deep brain structures of the striatum.[5,56] Wang et al. showed in a conditional transgenic HD mouse model that it will be crucial for drugs that lower HTT to reach in a sufficient amount not only the surface areas of the brain but also the striatum.[56] Although the infusions of antisense oligonucleotides to the cerebrospinal fluid (CSF) induced short-term non-selective HTT lowering in a nonhuman primate brain, the reduction of HTT was more extensive at approximately 50% in the cortex compared with 20% in the caudate nucleus.[57] The effect of HTT lowering was declining up to the termination of the study at three months. For siRNA therapeutics, >45% suppression of HTT in the nonhuman primate striatum was achieved in two studies using

chemically modified siRNAs administered stereotactically into the brain.[58,59] Comparison of these studies revealed that the duration of siRNA infusions into the brain strongly effects the duration of HTT suppression. Clinically more relevant shorter continuous three-day infusions lead up to 39-day therapeutic lowering till HTT suppression returned to zero.[59] An overall advantage of short-term "non-viral" induction of HTT lowering is a possibility to discontinue the treatment at any time. On the other hand, the results from nonhuman primate studies indicate that repeated administration either to the spinal cord or deep brain structures is required for persistent therapeutic effect. Periodical re-administrations present a lifelong burden for HD patients, which is higher if a treatment would need to be delivered directly into the striatum. The current clinical trial (https://clinicaltrials.gov/: NCT02519036) in humans will provide first insights into the safety and efficacy of CSF-delivered short-term therapies for HD that will require persistent re-administrations to the spinal cord.

Long-term HTT lowering

The limited drug accessibility to the human brain is one of the major challenges in the development of HD therapies and one-time delivery of a therapeutic that provides long-term benefit would have major advantages. Long-term HTT lowering can be realized by viral delivery of an expression cassette of RNAi precursors, such as miRNAs and short hairpin (sh)RNAs.[40,43,47] Similar to siRNA therapeutics, artificial miRNAs and shRNAs are designed to operate post-transcriptionally in a sequence-specific manner after being processed by the endogenous RNAi machinery (**Figure 1**).[60] shRNAs expressed from strong polymerase III promoters showed toxicity at high doses in mice.[61–63] In some cases, the shRNA toxicity correlated with high production of the passenger strand, a byproduct of RNAi processing, independent from the HTT mRNA silencing.[64] The latter was circumvented when the miRNA-based expression system was used instead.[64]

The most common delivery systems of RNAi expression cassettes are recombinant AAV or lentiviral (LV) vectors.[65,66] Whereas LV vectors integrate in the host genome, AAV genomes remain mostly episomal after transduction and therefore are less likely to cause random insertional mutagenesis and inadvertent activation of oncogenes.[67] AAVs are also know to effectively transduce non-dividing cells.[65,68] AAV vectors with increased cell- and tissue-specific tropism have been designed, and some AAV vectors exhibit anterograde and retrograde neuronal transport.[66,68] To date, AAV serotypes 1, 2, 5, 6, 8, 9, and recombinant human (rh)10 are the widely studied as delivery vectors for the central nervous system (CNS) indications.[68]

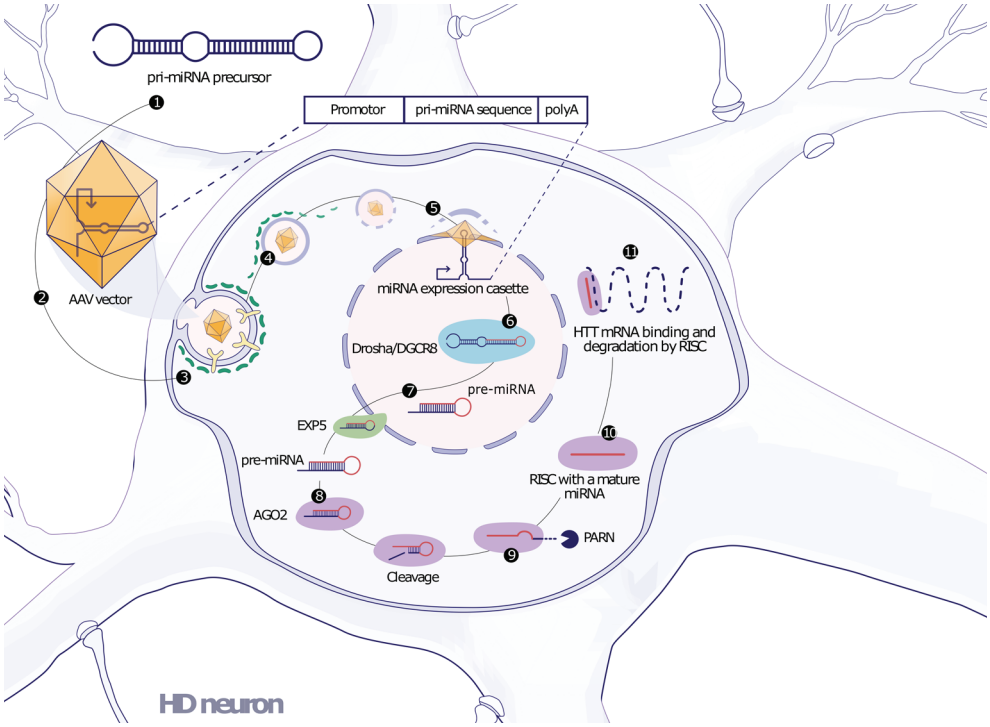

Figure 1. A therapeutic concept of an AAV-miRNA gene therapy using optimized miRNA precursors. An AAV-miRNA construct is generated by incorporating an expression cassette of a therapeutic miRNA precursor in an AAV vector (**1**). The resultant AAV-miRNA construct is delivered to the target cells, where it binds to cell-surface markers to induce endocytosis (**2**). Inside the cell, the AAV capsid is hydrolyzed (**3**) and AAV genome enters the nucleus (**4**). In the nucleus, the artificial miRNA is transcribed as a primary (pri)-miRNA precursor. The precursor folds into a typical stem-loop RNA structure and is further cleaved by DROSHA/DGCR8 complex at specific positions to render a pre-miRNA precursor (**5**). The pre-miRNA is exported out of the nucleus to the cytoplasm by Exportin 5 (EXP5) (**6**). In the cytoplasm, the precursor is recognized by RNA-induced silencing complex (RISC), from which Argonaute 2 (AGO2) enables artificial pre-miRNA cleavage, generation of the guide strand, and degradation of the passenger strand (**7**). The guide strand is further trimmed by poly(A)-specific ribonuclease (PARN) to render a mature miRNA therapeutic (**8**). RISC together with a mature miRNA bind to target HTT mRNA (**9**), which ultimately results in HTT protein lowering (**10**).

The efficacy and safety of RNAi-based gene therapies were first evaluated in HD rodent models and HTT lowering of 55% has been reported.[69] Following the initial report, more than twenty studies have been published using RNAi molecules as potential therapeutic compounds for HD treatment (**Supplementary table 1**).[70] Several studies reported inhibition of mutant HTT aggregate formation, and this reduced neuronal dysfunction in HD rats.[38,71,72] In a nonhuman primate study, AAV1-miRNA targeting rhesus HTT was magnetic resonance imaging (MRI)-guided stereotactically injected in the right and left putamen, which induced 45% HTT reduction in the mid- and caudal putamen without inducing neuronal degeneration, astrogliosis, or an immune response till the termination of the study at six weeks.[37] Similarly, injections of AAV2-shRNA targeting rhesus HTT induced well-tolerated 30% HTT reduction in the injected putamen measured at six months post injections.[73] More recently, AAV9-miRNA

unilateral injections in the striatum of the HD sheep induced 50-80% HTT protein lowering at six months post injections.[74] A dose-dependent distribution of AAV5-miRNA was achieved as well in a transgenic minipig model of HD (M.M.E. et al., unpublished data). Our study showed a significant lowering of human mutant HTT mRNA and protein in the striatum and more distal cortical areas at three months post injections (M.M.E. et al., unpublished data). Together, these data demonstrate a proof-of-concept in HD animal models, supporting the transition of RNAi-based gene therapy to the clinic.

Animal models and cell systems addressing key preclinical outcome measures relevant for the HTT lowering

Many HD animal models that represent the progressive degenerative phenotype of HD allow fast and clinically relevant assessments of novel treatment paradigms.[44–46,75,76] However, the different models represent only specific aspects of HD symptomatology,[44] No single model can be used to properly address all clinically relevant aspects of HTT lowering gene therapies. Therefore, preclinical development of an HTT-targeting gene therapy demands a combination of experiments including various HD cell and animal models addressing a broad spectrum of outcome measures.[28,77] In this section, we discuss HD animal and cell models based on their genetic and phenotypic aspects in relation to HTT lowering strategies.

Nematodes and fruit flies have been used for HD drug discovery, but we limit the scope of this review to the more relevant rodent and large animal models. HD animal models can be categorized based on the following genetic aspects: **1**) location of *HTT* gene insertion as knock-in or transgenic, **2**) construct engineering as full-length or partial mutant HTT, **3**) use of cDNA or genomic DNA, **4**) length of CAG expansion, **5**) use of the HTT or other promoters, and **6**) presence or absence of the host HTT.[44,78] The genetic background of HD animal models not only determines the phenotype, but also defines the availability for therapeutic targeting and subsequent translation of the approach to the clinic. As such, genetic therapies targeting directly DNA or RNA sequences of HTT require a presence of human target sequences in the animal model for preclinical testing. For instance, the efficacy of artificial miRNAs designed to target HTT exons closer to the 3' UTR cannot be assessed in HD animals expressing only the N-terminal fragment of human mutant HTT. Similarly, when addressing the allele-selective potential of miRNAs or antisense oligonucleotides based on a SNP in the *HTT* gene, the chosen animal model should not only carry the polymorphism but the SNP should be heterozygous. Ultimately, when selecting HD animal models, the conservation of target sequences between various species should be closely considered, as well as the effects of simultaneous targeting of the mutant and endogenous HTT. The latter is crucial for assessment of tolerability of non-selective HTT lowering, in which case the remaining levels of endogenous HTT are relevant.

Rodent HD models

Mice and rats are the most commonly tested species to address activity of therapeutic compounds in the preclinical development of HD and currently more than two dozens of rodent models have become available.[14,44,78] The most utilized mouse model of HD is a transgenic R6/2 developed by a random insertion of the human mutant HTT exon 1, originally containing 144 CAG repeats, into the mouse genome.[79,80] The gene expression is driven by the human HTT promoter and the mutant HTT is expressed at three quarters of the level of wild-type murine Htt, which has two copies.[79] Similar to juvenile HD patients, higher CAG repeat number manifests in these mice with earlier onset of disease, formation of HTT aggregates, rapidly progressive motor and cognitive deficits, and premature death. These characteristics distinguish this model from most of knock-in and full-length murine models, that have a less progressive phenotype.[81] Notably, somatic instability has been reported in R6/2 mice that manifest in variable CAG lengths.[82] This results in differential behavioral and neuropathological patterns that should be considered when testing HTT lowering therapies and comparing different studies. Another widely used transgenic HD mouse model N171-Q82 was generated with a HTT fragment containing 171 amino acids and 82 glutamines.[83] The gene is expressed from the mouse prion promoter as 20% of murine Htt. This model expresses less HTT and has shorter CAG repeats compared with R6/2 mice and subsequently it presents with later onset of symptoms.[81]

YAC128 and BACHD mice are well-known full-length mutant HTT models with 128 and 97 CAG repeats, respectively.[84–86] The transgenes are expressed from the human HTT promoter as 75% and 150% of Htt, respectively.[75,85,86] Both models show progressive motor, cognitive and psychiatric disturbances. The recent development of full-length transgenic humanized Hu128/21 (YAC128/BAC21) and Hu97/18 (BACHD/YAC18) mice by intercrossing YAC and BAC models with the Hdh-/- background now allows evaluation of the efficacy of new therapies in mice that do not express murine Htt and have two copies of the mutant human *HTT* gene.[87,88] Both Hu128/21 and Hu97/18 exhibit progressive motor, cognitive and psychiatric disturbances. Hu128/21 mice also show EM48 positive inclusions at nine months of age. These humanized HD mice offer unique opportunities to address the window of safety and efficacy of the non-selective HTT lowering as the murine HTT has been replaced by two human copies. Additionally, the humanized Hu128/21 model is also suitable for assessing the allele selectivity of HTT lowering agents targeting heterozygous SNPs or different CAG lengths. Hu97/18 is not a suitable model for assessing CAG-targeting therapeutics because of the mixed CAG-CAA tract.

The knock-in HD mouse models should represent, in theory, more accurately HD pathology since they are created by inserting a human HTT fragment with 50-200 CAGs into a part of or the entire murine Htt gene and the expression remains driven by the murine Htt promoter.[89,90] These mice are generated either homozygous or heterozygous for the human HTT fragment.[91] Overall, the behavioral deficits are reported to be not striking compared

to other murine models, which is important to consider when addressing the treatment efficacy.[14,81]

Notably, either weight gain or loss has been described in many murine HD models. R6/2 mice showed weight loss, whereas full-length murine HD models such as YAC128, BACHD, Hu97/18 and Hu128/21 mice demonstrate weight gain.[44,87,88] Hence, animal model-specific changes in body weight should be monitored since such changes could influence the results of motor and behavioral performances.

Whereas mouse models are plentiful, only three HD rat models have been described to date. The first HD rats were generated by a lentiviral delivery of a HTT fragment containing various CAG repeats.[15] These rats manifest with a rapid local neuropathology, including HTT aggregation, but do not display behavioral deficits.[15] Those models are suitable for evaluating suppression of neurodegeneration, as indeed was demonstrated for different miRNAs and shRNAs that lower HTT.[71,72] The second HD rat model is transgenic with a human HTT cDNA fragment containing 51 CAGs, whereas expression is under control of the rat Htt promoter.[92] These rats manifest with adult-onset neurodegeneration, motor, cognitive and behavioral deficits. Recently, a full-length transgenic BACHD rat model was generated that exhibits behavioral deficits, HTT aggregation and striatal neuronal loss.[93] In contrast to BACHD mice, these rats do not show increased body weight.

In summary, the progressive HD models that develop a severe neurodegeneration are suitable to evaluate the suppression of neuropathology related to HTT lowering. The slow models are suitable to study improvements of motor and behavioral deficits during a longer period of time after administrating the HTT lowering therapy. Therefore, a preclinical portfolio would ideally include studies with rapid- and slow-progression HD rodent models to gain a comprehensive understanding of the therapeutic efficacy, pharmacodynamics and potential toxicity of the treatment.

Large HD animal models

In the recent years, considerable efforts have resulted in the development of large HD animal models. These large animal models allow to study efficacy, safety, biomarker discovery, long-term HTT lowering in an animal model with a larger brain and closer similarities in immunophysiology to humans compared with rodents.[94] Two important models, the HD minipig and sheep, have been generated with the support of CHDI foundation.[95,96] The knock-in HD minipig was developed by an insertion of a fragment of mutant HTT encoding the first 548 amino acids (12 exons) containing 124 glutamines under control of the human HTT promoter.[96] The transgenic sheep was generated with a full-length human HTT cDNA encoding 73 glutamines under control of the human HTT promoter.[95] Having been recently developed, these large animal models do not yet show the HD phenotype, which is being carefully monitored. Thus, the use of large HD animal models for preclinical studies is currently restricted to direct measures related to lowering of mutant HTT. Large animal studies are

costly and lengthy as compared to rodent studies, and still need to be sufficiently powered to reach meaningful outcomes. Nevertheless, these studies should not be omitted from the preclinical development because they offer a much more realistic system regarding the delivery of gene therapy. Consequently, such studies are usually conducted after obtaining efficacy and safety signals in rodent models. Undoubtedly, a sufficient body of knowledge from the rodent studies is a requirement for a successful large animal study.

Cell systems modelling HD

During the last years, induced pluripotent stem cells (iPSCs) -derived neuronal cultures have become an accepted model of neurodegenerative diseases including HD, that enables early testing of the treatment efficacy and some aspects of the safety.[97–99] iPSCs are somatic cells, such as fibroblasts, reprogrammed to a pluripotent state by an addition of essential transcription factors, which can be subsequently differentiated into various cell types.[97] HD patient-derived iPSCs are being differentiated and matured into various neuronal lineages, astrocytes, or microglia and provide a unique platform to study the therapeutic efficacy of HTT lowering strategies in human patient cells.[100–103] Currently, the read-out of therapeutic efficacy in these HD neuronal cultures is the percentage of HTT lowering. However, several studies have been conducted to identify a functional phenotype as measurements of therapeutic efficacy, such as protein clearance, cell growth, adhesion, differentiation, survival, stress response. [reviewed 100] Dozens of transcribed polymorphisms have been found in various local HD patient cohorts that are in a linkage disequilibrium with the HD mutation, thus delineating different HD haplotypes.[34] This allows for an evaluation of HTT lowering compounds targeting a specific polymorphism present in different patient haplogroups. It should be noted that an inherent variability has been found among control iPSCs. Efforts are being made to generate more isogenic iPS lines by gene editing to more accurately define effects linked to the HD mutation.[100] Because gene expression signatures are often species-specific, these human cell-based models have become the preferred test system in parallel to animal studies to study therapy-induced neuronal protection as well as specific off-target silencing of other genes.[71,104]

Preclinical outcome measures for AAV-miRNA gene therapies

Translating preclinical studies to the clinic for an AAV-miRNA-based gene therapy involves establishing a therapeutic safety and efficacy window **(Figure 2)**. While designing preclinical studies, the emphasis should be placed on identification of a translatable framework that will provide sufficient understanding on the efficacy and safety for a given compound, as well as considering the overall financial cost and animal welfare.

Table 1: RNAi-based HTT lowering gene therapy approaches to date.

	Lowering/Improvement								
	Reference	**RNAi therapeutics**	**Vector**	**Human Target**	**HD Animal**	**Delivery**	**HTT RNA (%)**	**HTT protein or inclusions (%)**	**Motor or Behavioural**
HD rodents	Harper et al., 2005	shRNA (shHD2.1)	AAV1	Exon 2	N171-82Q	Striatum	51-55%	10-50%	Rotarod and Gait
	Rodriguez-Lebron et al., 2005	shRNA (siHUNT -1 and -2)	AAV5	Exon 1	R6/1	Striatum	78%	28%	Clasping
	Machida et al., 2006	shRNA (shEGFP)	AAV5	N/A	(eGFP) HD190QG	Striatum	N/A	82%	Rotarod and Clasping
	Huang et al., 2007	shRNA+Ad-HttQ103GFP	Ad	Exon 1	R6/2 (Ad-HttQ103GFP)	Striatum	N/A	Yes: in transduced areas, not quantified	N/A
	Franich et al., 2008	shRNA (shHD2	AAV1	Exon 1	Rat AAV1-HD70	Striatum	80-90%	50%	Forepaw use
	McBride et al., 2008	miRNA (mi2.4)	AAV1	Exon 2	CAG140	Striatum	70%	N/A	N/A
	Boudreau et al., 2009	miRNA (mi2.4)	AAV1	Exon 2	N171-82Q	Striatum	85%	Yes, not quantified	N/A
	Drouet et al., 2009	shRNAs	Lentivirus	Exon 1-4	Rat lenti-htt 171-82Q	Striatum	N/A	70-80%	N/A
	Stanek et al., 2014	miRNA	AAV1	N/A	YAC128	Striatum	45%	55%	Rotarod and Porsolt swim test
	Miniarikova et al.,2016	miRNA (miHTT)	AAV5	Exon1	Hu128/21	Striatum	N/A	80%	N/A
	Miniarikova et al.,2017	miRNA (miHTT)	AAV5	Exon1	Rat lenti-htt 171-82Q	Striatum	62%	98%	N/A
	McBride et al., 2011	miRNA (miHDS1)	AAV1	N/A	wild-type non-human primate	Striatum	45%	N/A	N/A
HD large animals	Grondin et al., 2012	shRNA (shHD5)	AAV2	N/A	wild-type non-human primate	Striatum	30%	45%	N/A
	Pfister et al., 2017	miRNA (miRHTT)	AAV9	Exon 48	HD sheep	Striatum	20-30%	49-81%	N/A
	Evers et al., 2018	miRNA (miHTT)	AAV5	Exon1	HD minipig	Striatum	40-73%	50-54%	N/A

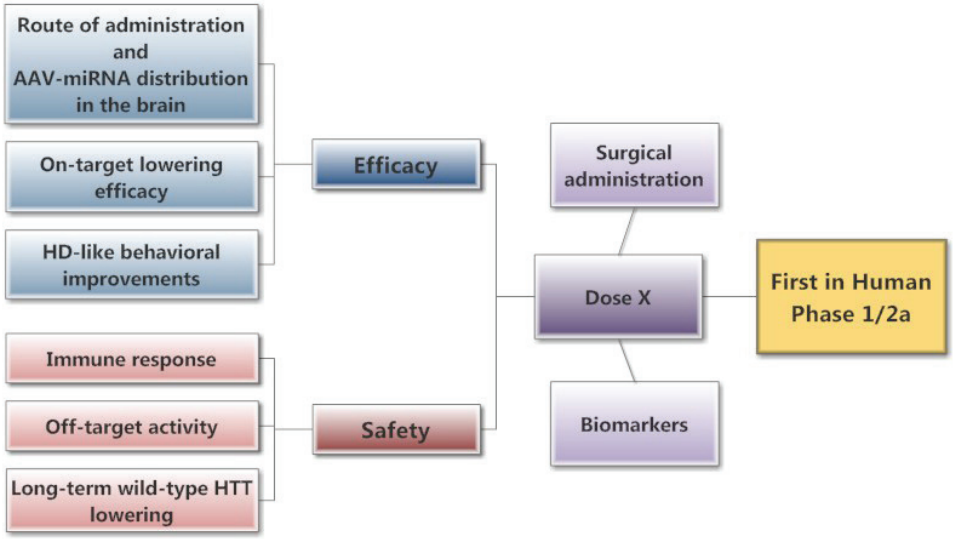

Figure 2. Preclinical measures of an AAV-miRNA gene therapy. The preclinical measures addressing the efficacy and safety aspects of an AAV-miRNA based HTT lowering gene therapy are outlined (in red and blue). Positive findings indicating a sufficient efficacy and no safety concerns demonstrated across multiple animal and cell models enable the selection of the adequate AAV dose and surgical procedure for the First in Human Phase 1/2a (in purple). Various biomarkers should be included in Human Phase 1/2a (in purple) and outlined as exploratory endpoints.

Evaluating efficacy for miRNA-based HTT lowering therapies

Many preclinical studies with potentially disease-modifying compounds have demonstrated a proof-of-principle in HD rodent models, but to date none of these putative treatments has yet successfully been translated to the clinic.[28] As discussed, several aspects of rodent models including their relatively small brain and simple anatomy, make a successful translation to the HD patient difficult. Adequate distribution is of paramount importance for AAV-miRNA gene therapy approaches, and this is dependent on the brain size and structure, the delivery vehicle, and the surgical procedure. All these require the use of a large animal model with a gyrencephalic brain similar to humans, as opposed to rodents with a lissencephalic brain.[45,105] The efficacy parameters of an AAV-miRNA gene therapy that should be explored in preclinical models are:

1. a route of administration to achieve optimal AAV distribution in the brain areas strongly affected by HD
2. mode of action or on-target lowering efficacy
3. HD-like behavioral improvements upon treatment

Route of administration and AAV-miRNA distribution in the brain

The development of AAV-based CNS deliveries has significantly progressed in recent years and multiple clinical studies have been initiated for Alzheimer's, Parkinson's, Canavan's

disease, late infantile neuronal ceroid lipofuscinosis, and Sanfilippo B syndrome.[106] The increased popularity of AAV vectors as drug vehicles for the treatment of neurodegenerative disorders reflects the long-term therapeutic benefits, the ability to transduce nondividing cells such neurons, and a relatively low immune response demonstrated in different models.[65,68] For CNS disorders, the therapeutic AAVs need to target specific brain areas, that are usually difficult to reach, or require a broad CNS coverage. In case of HD, sufficient AAV distribution in the striatum and cortex is crucial.[56] Several AAV serotypes showed efficacious transduction of neurons with different tropisms to immune cells of the brain such as astrocytes, microglia, and oligodendrocytes.[107] Given the brain complexity, the route of administration and the subsequent efficient vector genome delivery is one of the critical obstacles in a gene therapy for HD to eventually reach a clinical benefit.[108]

The three most frequently studied delivery routes of AAV vectors for CNS indications are: **A**) systemic administration through intravenous injection, **B**) direct infusions into the CSF, and **C**) local administration into the brain parenchyma. The CNS transduction efficacy and distribution of most AAV serotypes is being evaluated in large animals such as dogs, cats, minipigs and nonhuman primates (M.M.E. et al., unpublished data.[65,68,109–115] Here below, we will discuss the delivery routes in the HD context.

Intravenous injection

To reach the CNS upon the intravenous injection, AAV vectors carrying the expression cassette of the artificial miRNA precursors need to cross the blood-brain-barrier (BBB) or the blood-spinal cord barrier. These vascular barriers prevent most molecules from entering the CNS, thus providing protection against toxic or infective agents in the blood, as well as maintaining the chemical composition of the interstitial fluid.[116] Most of AAVs are not able to cross the BBB, with an exception of AAV9 which transduces the brain following intravenous injections.[117] The natural capacity of AAV9 to cross the BBB has been greatly augmented by further engineering the capsid, which led to a 40-fold and greater transduction in the murine brain when compared to the native AAV9 serotype.[118,119] Disappointingly, a recent results in marmosets did not recapitulate the enhanced transduction efficacy of these engineered capsids.[120] It still needs to be determined in other large species whether the engineered AAV capsids, when injected intravenously, specifically transduce the relevant target structures that are affected in HD. Thus, the intravenous delivery has an advantage of a low invasive nature, but the translational feasibility is challenging as extremely high doses would be necessary to therapeutically target the deeper structures in the CNS.

Direct infusion in the CSF

AAV vectors can be delivered to the brain by direct infusions in the CSF via intracerebroventricular (ICV) infusions into the lateral ventricles, IT injections in the spinal canal, or by direct administration to the cisterna magna or subarachnoid space. [68,73,109–111,121–123]

To reach the target brain structure of HD upon ICV or IT injections, the AAV needs to pass the ependymal cell layer surrounding the ventricular system or the pia mater, respectively. AAV serotypes 2, 4, 5, and 9 mainly transduce the ependymal cell layer once injected ICV, with a limited penetration into the brain parenchyma.[124] Most of studies using IT injections of various AAV serotypes have been conducted in rodents with a common outcome: the spinal cord is generally effectively transduced, but the vectors poorly reach deeper brain structures such as the striatum.[125–127] Therapeutic administration of AAV vectors to the CSF requires higher doses compared to a local administration and therefore, it may be associated with a higher likelihood of causing an immune reaction. Additionally, ICV and IT deliveries have been reported to cause a vector leakage into the periphery, limiting the clinical efficacious dose in the required brain structures.[68,111,122] On the other hand, in the case of HD, vector leakage is not unwanted *per se*, as (mutant) HTT is widely expressed outside the CNS and may be the cause of peripheral signs of the disease.[128] If IT administration of AAV vectors would result in sufficient striatal transduction, this would offer a major advantage for the clinical development of therapeutic products for HD, and promising results have been reported using AAV5 and AAV9 in nonhuman primates.[112,122]

Intracranial parenchymal administration

Despite recent improvements in an AAV-delivery following systemic or IT administration, at present, direct delivery to the parenchyma will most likely be preferred for HD-disease modifying therapies, because this method ensures sufficient transduction of deep brain structures. To date, more than ten clinical trials have been conducted or are ongoing using direct parenchymal injections of gene therapy products to the brain. [125,129–135] These studies have built on experience with therapeutic electrophysiological procedures. For example, the implantation of electrodes in the pallidus for deep brain stimulation was safe in an already degenerated HD brain.[136] None of the trials completed to date have demonstrated significant clinical benefit, possibly related to the low amount of vector used in the initial trials.[108] Nevertheless, these clinical studies did show that direct injections subcortically, in the substantia nigra or in the putamen, are safe and not associated with serious adverse events.

Optimizations of intracranial parenchymal deliveries show promises as the convection-enhanced delivery (CED) demonstrated an efficacious transmission of molecules to the brain that do not diffuse well.[137] In contrast to diffusive therapies, which are limited by concentration gradients, CED resulted in a high local concentration of drugs with a low systemic absorption.[137] For clinical applications, one or more catheters are stereotactically positioned using imaging methods into the interstitial space of the brain.[138] An infusion pump that is connected to the catheter creates a pressure gradient and drives the drug flow by replacing the extracellular fluid. To increase the safety and efficacy of CED, a reflux-resistant cannula has been developed.[139] CED resulted in improved distribution patterns

of AAV serotypes 1, 2, 5, 8, and 9 in the brain of rodents and nonhuman primates after direct injections into the brain.[109,110,140,141] This technique also showed improved diffusions throughout the brain in the clinical trial for Parkinson's disease and is currently being tested in other trials.[142]

Notably, the viral spread upon a local delivery is dependent on the intracellular transport, which occurs in either the anterograde or retrograde direction along axons.[107,143] The extent of axonal transport and distal transduction differs between the AAV serotypes and the mechanisms responsible for this variability are not clear yet.[107] For the HD treatment, the cortico-striatal circuitry in humans and large animals allows for transductions of distal areas, such as the cortex, from the striatal injection sites.[109,143] To date, all AAV1-, AAV5-, and AAV9-miRNA or AAV2-shRNA-based preclinical studies in HD animal models applied direct injections into the striatum to show a widespread AAV distribution in the CNS (M.M.E. et al., unpublished data).[39,64,72,104,144]

Measurements of on-target lowering efficacy

miRNA-based lowering strategies are designed to lower HTT mRNA levels and thereby to reduce the overall mutant HTT protein. Already in the early preclinical development, it is essential to establish the on-target activity of therapeutics by measuring their effects on HTT mRNA and protein levels. Usually, the initial assessment of a mode of action of therapeutic miRNAs is addressed using *in vitro* cell and reporter systems.[39,144] As discussed, a further extrapolation of observed HTT lowering into both slow- and rapid-progression rodent models is necessary to assess pharmacodynamics, aggregation, functional signs, and symptoms. In 2005, Harper et al. showed for the first time the feasibility of lowering human HTT in N171-82Q mice after injections of AAV1-shRNA vectors into the striatum.[69] In subsequent studies, R6/1, R6/2, CAG140, lenti-htt171-82Q, lenti-htt853-82Q and Hu128/21 rodent models have been successfully used to evaluate the efficacy of RNAi therapeutics.[36,39,64,72,145,146] In contrast to large HD animal models, HD rodents offer measurements of lowering HTT aggregation as an HTT-dependent readout.[69,72,146] The final assessment of a mode of action should be evaluated in a large HD animal model since the physiological, neurological and genetic background relates closer to the human. Although large HD animal models have been available for several years, only two studies have been described so far (M.M.E. et al., unpublished data).[74] A single AAV5-miRNA or AAV9-miRNA administration into a minipig or sheep striatum resulted in the mutant HTT mRNA and protein lowering up to 75%-80% in injected structures lasting at least three and six months post-injections, respectively. Altogether, these data highlight the importance of a careful characterization of on-target lowering efficacy in various HD models.

HD-like behavioral improvements in rodents

Deterioration of motor, cognitive, and psychiatric disturbances are a hallmark of progressive HD and studying these abnormalities in animals is important as it allows an evaluation of the clinical benefit for a specific HTT-targeting intervention.[44] Since the current HD animal models are quadrupeds or invertebrates, the correlations between the behavioral changes in these models and humans should be carefully evaluated nevertheless, a large battery of behavioral changes reported in HD rodents can be used to study efficacy of an AAV-miRNA gene therapy.[Reviewed in 44] To study improvements of motor functions, HD rodents are usually assessed using rotarod, climbing performance, gait analysis, and the balance beam test. [36,69,147] To measure cognitive improvements, learning capacity and memory of rodents are being addressed,[44] and anxiety-like and depression-like changes are used to study improvements in psychiatric measures.[44] Noteworthy, positive findings in behavioral improvements are usually challenging to conclude from a small subset of studied measures and a difficulty is added if the improvement window is not large enough to evaluate a dose response. Therefore, behavioral studies should be efficiently powered and include multiple measures that offer a highest outcome difference between the HD and control animals. Unfortunately, large HD animal models do not -yet- show HD-like symptoms, which precludes their use to study disease-specific functional, behavioral or psychiatric complications.

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SCOPE OF THE THESIS

HD is a fatal polyglutamine (polyQ)-associated monogenic neurodegenerative disorder with no disease-modifying treatment currently available. The underlying cause of HD pathology is an expansion of CAG repeats in the huntingtin (*HTT*) gene, translated into a polyQ region, and generating a toxic mutant HTT protein. Therapeutic strategies that are designed to lower the production of the mutant HTT are currently of primary interest for the scientific community and industry.

This thesis focuses on *in vitro* and *in vivo* studies contributing to the preclinical development of the micro (mi)RNA-based HTT lowering gene therapy to treat HD. It includes the pioneering preclinical studies of the lead AAV5-miHTT construct (AMT-130), which is planned to enter the clinic in 2019 (www.uniQure.com). More specifically, we have developed and preclinically tested therapeutic miRNAs, delivered and expressed from an adeno-associated viral serotype 5 (AAV5) vector, targeting HTT transcripts to reduce the formation of toxic HTT protein. The results published in this thesis contribute to the translation of the AAV-delivered-miRNA-based gene therapy approach from preclinical to the clinical setting.

In chapter 1, we summarize the current knowledge on the promising HTT lowering therapies as a treatment for HD with a focus on the miRNA-based gene therapy. We outline the animal and cell systems modelling HD that allow for the preclinical testing of HTT lowering therapies, emphasizing that there is no single model that can be used to fully address all clinically relevant aspects of HD treatments. We discuss the preclinical outcome measures of efficacy suitable for an AAV-miRNA-based gene therapy that support the translation to the clinic.

To develop an HD therapy that continuously lowers HTT transcripts and thereby reduces the mutant HTT protein, **in chapter 2**, we have designed dozens of therapeutic miRNAs targeting different regions in human HTT transcripts and analysed their efficacy *in vitro* and *in vivo*. Two approaches have been addressed. The non-selective HTT lowering was achieved by targeting sequences located on both HTT alleles. We showed efficacious mutant HTT knock-down induced by the miHTT construct delivered by the AAV5 vector *in vitro* and in the striatum and cortex of the humanized Hu128/21 HD mouse model. Furthermore, we have evaluated the efficacy and allele-selectivity of therapeutic miRNAs targeting CAG repeats and heterozygous single nucleotide polymorphisms (SNPs) in linkage disequilibrium with the mutant HTT allele. In this study, we also evaluated the role of pre-miRNA scaffolds in the efficacy and preliminary safety of therapeutic miRNA constructs and identified the pre-miR-451 precursor suitable for the miRNA-based gene therapy to treat HD.

To further address the efficacy in suppressing the HD-like neuropathology, **in chapter 3,** we performed a second preclinical study with the selected miHTT construct targeting both HTT alleles, processed from the pre-miR-451 precursor, and delivered by AAV5 vectors

(named as the AAV5-miHTT throughout the thesis) in the striata of the lentiviral HD rat model. We have shown a strong reduction of HTT aggregates, which resulted in a reduction of HD-like neuronal dysfunction. Moreover, we demonstrated the allele-specific HTT lowering by a therapeutic miRNA targeting T isoform of SNP rs362331 in exon 50. These results supported further testing of AAV5-miHTT construct in a minipig model of HD, which is not in the scope of this thesis.

The general concept of miRNAs being mainly active in the cytoplasm has been recently challenged, with evidence demonstrating the nuclear activity of cellular miRNAs. This concept offers new opportunities for miRNA-based therapies to target and lower disease-related transcripts in the nucleus and cytoplasm, simultaneously. In case of HD, this might be particularly of interest since there are data showing HTT RNA-associated toxicity in HD mice. To investigate the potential of artifical miRNAs to induce HTT lowering in the nucleus, **in chapter 4,** we have designed and tested several miRNAs selectively targeting a four-nucleotide deletion in HTT intron 22 (ΔACTT). We have shown a first demonstration that mature therapeutic miRNAs delivered by an AAV5 vector can shuttle to the nucleus to induce lowering of endogenous mutant HTT in induced pluripotent stem cells (iPSCs)-derived HD neuronal cultures.

In preparations for a clinical trial, it will be important to identify pharmacokinetic/pharmacodynamics (PK/PD) modeling measures and biomarkers from easily obtainable biofluids that would provide correlations between the drug dosing, concentration, and efficacy in HD patients. To identify a source of quantifiable PK/PD measures which would signal the presence of an active therapeutic miRNA in the patient brain, **in chapter 5,** we have investigated exosomes as potential carriers of therapeutic miRNAs, released from iPSC-derived HD neuronal cultures transduced with the AAV5-miHTT vector. We confirmed the presence of mature miHTT molecules in the vesicles secreted from these cells, prompting further investigations using large animals, which is not in the scope of this thesis.

To summarize the current knowledge on the preclinical development for the miRNA-based gene therapy approaches for HD, **in chapter 6,** we discussed additional aspects of the translation of HTT lowering gene therapy to the clinic. We outline the safety measures for the miRNA-based gene therapy and highlight the current need for quantifiable biomarkers to monitor the AAV5-miHTT treatment response and outcomes. We emphasize the intriguing opportunity to investigate exosomes as potential carries of PK/PD measures. We also provide an overview on the current knowledge for the nuclear transcript lowering by cellular miRNAs. Ultimately, we comment on the future perspectives of the miRNA-based lowering gene therapy for HD.