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, which gives rise to a toxic mutant HTT protein. There have 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 microRNA (miRNA)-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 existing knowledge in preclinical models.

Huntington’s disease (HD) is the most common autosomal dominant neurodegenerative disorder, with a prevalence rate of 10 in 100,000 individuals worldwide. 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. Carriers of 36–39 CAG repeats show reduced penetrance. Mutant HTT protein causes neuropathology affecting the entire brain, with medium spiny neurons of the striatum being particularly vulnerable at early stages. The clinical symptoms include progressive motor, cognitive, and psychiatric disturbances. 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. The age of motor onset is typically in mid-life with a median survival of 15–18 years. Occasionally, HD manifests in juveniles with a typical severe phenotype due to the very long CAG expansion. 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. A consistent key feature of HD pathogenesis is the aggregation of mutant HTT protein in different conformations and at various cellular localizations. 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, and blocking polyQ-mediated aggregation has neuroprotective effects in transgenic HD mice. 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. Apart from the HTT aggregation, a formation of mutant HTT fragments of different lengths has been implicated as an essential event in HD pathology. 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. Short HTT exon 1 fragments can be translated from aberrant splice variants in knockout HD mice, and these fragments have also been localized in the brain of HD patients post mortem. 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.

Early therapeutic HD strategies focused on 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. 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

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the induction of HD pathogenesis, HTT lowering is currently considered to be the main therapeutic objective for HD.\textsuperscript{29} Even after the onset of symptoms, a conditional blockade 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.\textsuperscript{29} An effective therapeutic HTT-lowering approach would require long-term HTT suppression in wide areas of the brain, which can be achieved using gene therapy technologies.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{31,32,34,35} Therefore, a majority of the most advanced projects focus on the non-selective HTT lowering.\textsuperscript{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.\textsuperscript{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.\textsuperscript{20,40,41} One of the most advanced long-term HTT-lowering strategies is a microRNA (miRNA)-based gene therapy that comprises a single administration of an adeno-associated viral (AAV) vector delivering an expression cassette of a therapeutic miRNA precursor.\textsuperscript{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 is clinically relevant and supports 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.\textsuperscript{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 \textit{in vitro} and \textit{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 rationale 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.\textsuperscript{29} Here, we will discuss these HTT-lowering therapies in 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.\textsuperscript{20} Antisense oligonucleotides and small interfering RNAs (siRNAs) 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.\textsuperscript{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.\textsuperscript{52–55}

Although HTT is widely expressed throughout the brain, the neuronal damage is more prominent within the corticostriatal circuitry involving the cortex and deep brain structures of the striatum.\textsuperscript{5,56} Wang et al.\textsuperscript{56} 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. 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.\textsuperscript{57} The effect of HTT lowering was declining up to the termination of the study at 3 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.\textsuperscript{58,59} Comparison of these studies revealed that the duration of siRNA infusions into the brain strongly affects the duration of HTT suppression. Clinically more relevant shorter continuous 3-day infusions lead up to 39-day therapeutic lowering until HTT suppression returned to zero.\textsuperscript{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 the repeated administration either to the spinal cord or deep brain structures is required for a 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**

Limited drug accessibility to the human brain is one of the major challenges in the development of HD therapies, and one-time administration 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 RNAs (shRNAs). 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). shRNAs expressed from strong polymerase III promoters showed toxicity at high doses in mice. 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. The latter was circumvented when the miRNA-based expression system was used instead.

The most common delivery systems of RNAi expression cassettes are recombinant AAV or lentiviral (LV) vectors. AAVs are also known to effectively transduce non-dividing cells. AAV vectors with increased cell- and tissue-specific tropism have been designed, and some AAV vectors exhibit anterograde and retrograde neuronal transport. To date, AAV serotypes 1, 2, 5, 6, 8, and 9 and recombinant human (rh)10 are widely studied as delivery vectors for the CNS indications. The efficacy and safety of RNAi-based gene therapies were first evaluated in HD rodent models, and HTT lowering of 55% has been reported. Following the initial report, more than twenty studies have been published using RNAi molecules as potential therapeutic compounds for HD treatment (Table S1). Several studies reported inhibition of mutant HTT aggregate formation, and this reduced neuronal dysfunction in HD rats. In a nonhuman primate study, AAV1-miRNA targeting rhesus HTT was 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 until termination of the study at 6 weeks. Similarly, injections of AAV2-shRNA targeting rhesus HTT induced well-tolerated 30% HTT reduction in the injected putamen measured at 6 months post injections. More recently, AAV9-miRNA unilateral injections in the striatum of the HD sheep induced 50%–80% HTT protein lowering at 6 months post injections. 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 3 months post injections. Together, these data demonstrate a proof-of-concept in HD animal models, strongly supporting the transition of RNAi-based gene therapy to the clinic.

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**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 miRNA (pri-miRNA) precursor. The precursor folds into a typical stem-loop RNA structure and is further cleaved by the 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, binds to the target HTT mRNA (9), which ultimately results in HTT protein lowering (10).
Animal Models and Cell Systems Addressing Key Preclinical Outcome Measures Relevant for HTT Lowering

Many HD animal models that represent the progressive degenerative phenotype of HD allow fast and clinically relevant assessments of novel treatment paradigms. However, the different models represent only specific aspects of HD symptomatology. 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. 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 knockin 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. 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 directly targeting 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 microRNAs 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 microRNAs 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 dozen rodent models have become available. 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. 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. Similar to juvenile HD patients, a 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 the knockin and full-length murine models, which have a less progressive phenotype. Notably, somatic instability has been reported in R6/2 mice that manifest in variable CAG lengths. 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 an HTT fragment containing 171 amino acids and 82 glutamines. 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.

YAC128 and BACHD mice are well-known full-length mutant HTT models with 128 and 97 CAG repeats, respectively. 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. Both Hu128/21 and Hu97/18 exhibit progressive motor, cognitive, and psychiatric disturbances. Hu128/21 mice also show EM48-positive inclusions at 9 months of age. These humanized HD mice offer unique opportunities to address the window of safety and efficacy of the non-selective HTT lowering because 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 knockin HD mouse models should represent, in theory, HD pathology more accurately because 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. These mices are generated either homozygous or heterozygous for the human HTT fragment. 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.

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. Hence, animal-model-specific changes in body weight should be monitored because 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 rat models were generated by a lentiviral delivery of an HTT fragment containing various CAG repeats.
These rats manifest with a rapid local neuropathology, including HTT aggregation, but do not display behavioral deficits. Those models are suitable for evaluating suppression of neurodegeneration, as indeed was demonstrated for different miRNAs and shRNAs that lower HTT. The second HD rat model is transgenic, with a human HTT cDNA fragment containing 51 CAGs, whereas the expression is under control of the rat Htt promoter. These rats manifest with adult-onset neurodegeneration and 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. 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 recent years, considerable efforts have resulted in the development of large HD animal models. These large animal models allow study of efficacy, safety, biomarker discovery, and long-term HTT lowering in an animal model with a larger brain and closer similarities in immunophysiology to humans compared with rodents. Two important models, the HD minipig and sheep, have been generated with the support of the CHDI Foundation. The knockin 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. The transgenic sheep was generated with a full-length human HTT cDNA encoding 73 glutamines under control of the human HTT promoter. 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 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 Modeling HD
During the last few years, induced pluripotent stem cells (iPSCs)-derived neuronal cultures have become an accepted model of neurodegenerative diseases, including HD, which enables early testing of the treatment efficacy and some aspects of the safety. 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. 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. Currently, the readout 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, and stress response. 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. 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 iPSC lines by gene editing to more accurately define effects linked to the HD mutation. 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.

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). When 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.

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 been successfully translated to the clinic. 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, delivery vehicle, and 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.

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) a mode of action or on-target lowering efficacy, and (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 lipofuscinos, and Sanfilippo B syndrome. 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 as neurons, and a relatively low immune response demonstrated in different models. For CNS disorders, the therapeutic AAVs need to target specific brain areas, which are usually difficult to reach, or require a broad CNS coverage. In the case of HD, sufficient AAV distribution in the striatum and cortex is crucial. Several AAV serotypes showed efficacious transduction of neurons with different tropisms to immune cells of the brain, such as astrocytes, microglia, and oligodendrocytes. Given the brain complexity, the route of administration and subsequent efficient vector genome delivery is one of the critical obstacles in a gene therapy for HD to eventually reach a clinical benefit.

The three most frequently studied delivery routes of AAV vectors for CNS indications are (1) systemic administration through intravenous injection, (2) direct infusions into the CSF, and (3) 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). Here, we will discuss the delivery routes in the HD context.

Intravenous Injection. To reach the CNS upon 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. Most of the AAVs are not able to cross the BBB, with the exception of AAV9, which transduces the brain following intravenous injections. 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. Disappointingly, recent results in marmosets did not recapitulate the enhanced transduction efficacy of these engineered capsids. 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 because 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 ICV infusions into the lateral ventricles, IT injections in the spinal canal, or by direct administration to the cisterna magna or subarachnoid space. To reach the target brain structure of HD upon intracerebroventricular (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. Most of the 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. Therapeutic administration of AAV vectors to the CSF requires higher doses compared to 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. On the other hand, in the case of HD, the vector leakage is not unwanted per se because (mutant) HTT is widely expressed outside the CNS and may be the cause of peripheral signs of the disease. 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.

Intracranial Parenchymal Administration. Despite recent improvements in the 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. 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. 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. 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 promise as the convection-enhanced delivery (CED) demonstrated an efficacious transmission of molecules to the brain that do not diffuse well. In contrast to diffusive therapies, which are limited by concentration gradients, CED resulted in a high local concentration of drugs with low systemic absorption. For clinical applications, one or more catheters are stereotactically positioned using imaging methods into the interstitial space of the brain. 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. 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.\textsuperscript{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.\textsuperscript{142}

Notably, the viral spread upon a local delivery is dependent on the intracerebral transport, which occurs in either the anterograde or retrograde direction along axons.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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).\textsuperscript{19,64,72,104,144}

**Measurements of On-Target Lowering Efficacy**

miRNA-based lowering strategies are designed to lower HTT mRNA levels and thereby reduce the overall mutant HTT protein. Already in 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.\textsuperscript{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.\textsuperscript{69} showed for the first time the feasibility of lowering human HTT in N171-82Q mice after injections of AAV1-shRNA vectors into the striatum. 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.\textsuperscript{6,39,64,72,145,146} In contrast to large HD animal models, HD rodents offer measurements of lowering closer to that of 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).\textsuperscript{74} A single AAV5-miRNA or AAV9-miRNA administration into a minipig or sheep striatum resulted in the mutant HTT mRNA and protein lowering of up to 75%–80% in injected structures lasting at least 3 and 6 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 because it allows an evaluation of the clinical benefit for a specific HTT-targeting intervention.\textsuperscript{44} Because 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.\textsuperscript{44} To study improvements of motor functions, HD rodents are usually assessed using rotarod, climbing performance, gait analysis, and the balance beam test.\textsuperscript{36,69,147} To measure cognitive improvements, learning capacity and memory of rodents are being addressed,\textsuperscript{64} and anxiety- and depression-like changes are used to study improvements in psychiatric measures.\textsuperscript{64} Noteworthy, positive findings in behavioral improvements are usually challenging to conclude from a small subset of studied measures and 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 preclude their use to study disease-specific functional, behavioral or psychiatric complications.

**Safety Issues Specific for AAV-Delivered miRNA-Based HTT-Lowering Therapies.** Safety is a major aspect of drug development, and,
because of the long-term and irreversible nature of gene therapy, evaluation of short- and long-term toxicity is critical. Besides the routine safety studies that will not be discussed in this review, an AAV-miRNA gene therapy should be appropriately evaluated to (1) rule out a possible immune response to the AAV capsid or vector DNA, (2) exclude potential off-target activity by overexpression of the therapeutic miRNA precursors, and (3) demonstrate tolerability to long-term HTT lowering.

**Immune Response**

AAV capsids as well as therapeutic DNA expression cassettes can provoke immune responses and novel gene therapy approaches expressing miRNA precursors needed to address the potential of activating the immune system in the preclinical studies. Neuro-physiology and immunophysiology of HD animal models should be taken into account when designing such preclinical studies, especially because neurodegenerative disorders are known to induce a primed immune response, which is a result of a cascade of events following the presence of a toxic protein. HD patients were shown to have mutant HTT-related activation of microglial cells in the striatum, with cytokine upregulation in the body fluids. Preclinical models of HD are also characterized by abnormal immune activation and in several HD mouse models microglial activation in the striatum has been demonstrated. Transgenic HD minipigs display changes in the cytokine levels in the CNS, CSF, and serum as compared to healthy littermates. Therefore, these animal models provide a great opportunity to investigate a potential immune reaction to an AAV-miRNA gene therapy in the HD background.

AAV1, AAV2, and AAV5 preferentially transduce neurons and astrocytes after intracranial injection, whereas AAV9 more efficiently transduces astrocytes after intravenous delivery. This cellular tropism has qualitative and quantitative consequences on the activation of immune response in the CNS. Intraparenchymal delivery of AAV1-miRNA to nonhuman primates has resulted in a dose-dependent mild microglial response and astroglialosis around the tip of injection, very likely due to a local injury caused by the needle. Likewise, cytokines released from astrocytes and microglia were found to be transiently upregulated after intracranial delivery of the AAV5-miRNA gene therapy in a transgenic HD minipig model. Next to local inflammatory and immune responses in the putamen, cell-mediated and humoral responses should be carefully evaluated to conclude that the AAV-miRNA gene therapy results in a constitutive activation of a peripheral immune response. A recent publication has shown a transient mild cytokine increase in the CSF after administrating the AAV5-miHTT in tgHD minipigs for up to 2 weeks (M.M.E., unpublished data). The cytokine increase returned to zero and remained as such until the termination of the study at 3 months. Therefore, the results from the above studies suggest a possibility of transient immune activation in the periphery shortly after the drug administration, without causing adverse events.

**Prediction and Evaluation of miRNA On- and Off-Target Activities**

A general safety concern for RNAi therapeutics is unwanted side effects caused by expressing artificial miRNA precursors that lower HTT. These can be categorized as (1) negative on-target effects, which can result in dysregulation of genes due to lower HTT levels, and (2) off-target effects caused by nonspecific binding of the miRNA to other mRNAs, resulting in a prevention of translation. There are cases showing that overexpression of shRNAs and miRNAs can cause toxicity and off-target activity caused by saturation of the RNAi machinery related to cellular processing of therapeutic precursors or resulting from miRNA target sequence similarity with other genes. In general, miRNA-induced gene silencing effect is based on 7 to 8 nucleotide homology between the so-called “seed” region of the miRNA and the 3’ UTR of the gene. To date, all miRNAs that showed preclinical efficacy to lower the HTT mRNA were designed in silico and vary in the length and sequence composition. The roles of specific miRNA sequences in the context of efficacy in target lowering are heavily studied, but so far the exact formulas enabling the design of highly efficient therapeutic miRNAs are largely missing. In the case of HTT silencing, the artificial miRNAs are being designed with a complete homology to induce sufficient HTT silencing, but they might also bind and lower the expression of other genes. Off-targeting can be minimized by adopting a stringent design filter for both guide and passenger strands of the RNAi effector molecules. This approach has been validated for RNAi therapeutics targeting HTT, in which the off-target effect was significantly minimized by avoiding partial complementarity of siRNAs to 3’ UTR regions of nontargeted genes, whereas a strong lowering of HTT was maintained. Notably, miRNAs are more accurately processed by Dicer compared with shRNAs, which increases their specificity to the target region. Importantly, a specific precursor backbone was recently identified for the artificial miRNA and shRNA design that does not generate a passenger strand by avoiding Dicer processing, and thus, a possibility of off-target silencing due to the passenger strand can be eliminated.

The analysis of potential off-target activities caused by the RNAi therapeutics is rather complex in the HD background. HTT has been implicated in the transcriptional regulation, and changes in gene expression patterns have been detected in HD animal models. Off-target effects may be predicted by seeking for genomic sequences with partial homology to the artificial miRNA targets and addressing the potential gene expression changes in HD animal and cell models.

**Long-Term Wild-Type HTT Lowering**

Most HTT-lowering therapies do not discriminate between HTT alleles, and the potential toxicity of long-term HTT lowering is therefore a major safety concern. Wild-type HTT is highly conserved and has important physiological functions in neuronal development, axonal transport, transcriptional regulation, and protection from cell death. The long-term consequences of interfering with these processes in the adult HD context is largely unknown. Complete
elimination of wild-type HTT is embryonically lethal in mice and negatively impacts adult neuronal function by reduction of neurotrophic factors, hampered mitochondrial trafficking, and increased production of reactive oxygen species. Thus, silencing of the wild-type HTT must be done, with recognition that residual gene expression should be maintained at safe levels. Heterozygous Htt knockout mice are phenotypically normal and humans with only one copy of HTT (50% reduction of normal HTT production) show no abnormal behavioral deficits. Recent studies have demonstrated that depletion of Htt in adult neurons is non-deleterious and HTT without N-terminal region is functional, suggesting that silencing of HTT expression can be achieved within a certain therapeutic window in an adult human.

A suitable system to study the long-term effects of HTT lowering would be a large animal brain. Current safety studies are lacking measurements beyond 6 months of post injections (M.M.E. et al., unpublished data). The evaluation of consequences of long-term (>6 months) 50% HTT lowering in large animals should be included in the preclinical protocol before entering the clinic.

In summary, results from small and large HD animal studies suggest that a local (striatal), long-term (in months), and partial inactivation of endogenous HTT in adult brains can be achieved without important toxicity. Notably, in most rodent models except for the humanized mice, the safety of wild-type HTT silencing cannot be studied because the human HTT is expressed on the background of the murine Htt copies and in order to silence both HTT-expressing genes, the target region needs to have enough sequence conservation. Further investigations are needed to evaluate if long-term non-selective HTT lowering affects neuronal function and whether compensatory changes in the brain occur to prevent abnormal neurological functions.

**Additional Aspects of Translation of HTT-Lowering Gene Therapy to the Clinic**

Next to the required toxicology studies, the conducted preclinical studies are important for designing phase I/IIa clinical trials, particularly for (1) selecting the initial clinical dose, with a subsequent escalation in humans, and (2) identification of quantifiable biomarkers that would signal that the therapeutic is present and effective in the brain.

**Dose Finding**

Dose finding in gene therapy studies is complex because it is not ethical to expose patients to either a non-active dose or a potentially toxic dose. The initial dose for a phase I/IIa trial is calculated based on a conversion factor that translates results from preclinical animal and cell studies to the human brain. Several variables should be accounted for, including the differences in size and volume between animal and human brains, the spread of the volume delivered, and the AAV genomic copy number. Our studies showed linear correlations between the injected AAV vector DNA copies, miRNA expression, and HTT-lowering efficacy, and we demonstrated that a minimal number of miRNA molecules per cell is required to reach 50% mutant HTT mRNA reduction (M M.M.E. et al., unpublished data). Nonetheless, the translation of preclinical results acquired in a relatively small animal brain to a large HD patient brain remains challenging. For instance, intra-striatal injections of AAV-miRNA gene therapy in small murine HD brains resulted in a widespread AAV distribution and HTT lowering throughout the brain. On the other hand, intra-striatal delivery of AAV-miRNA gene therapy in nonhuman primates and transgenic HD minipigs resulted in a strong local transduction using lower viral doses and a more widespread AAV transduction and HTT lowering with an increasing viral load (M.M.E. et al., unpublished data). AAVs preferentially make use of anterograde transport, whereas retrograde transport can be achieved with higher concentration of the virus, suggesting that distribution patterns can be dose dependent. An additional layer of complexity is added by the HD neuropathology with progressive white matter degeneration that is thought to reduce connectivity between the putamen and motor cortex and thus influences the viral spread. This further underlines the importance of proper dose-range finding studies in large HD animal models to close the gap between preclinical research in HD rodents and clinical research in humans.

**Pursuits for Valid Clinical Biomarkers**

The therapeutic goal of an AAV-miRNA gene therapy is to delay disease onset and/or slow down disease progression. The ultimate success of a clinical trial strongly depends on a robust validation of on-target efficacy and proof-of-principle. This is difficult to achieve without engaging and sampling the treated biochemical environment. In the case of HD, brain biopsies are possible but highly risky. Not fully described HD natural history and heterogeneity of clinical symptoms present major obstacles for identifying clinical efficacy end points for HD treatment. Characterization of quantifiable and treatment-responsive biomarkers that correlate with HD neurodegeneration is heavily investigated. Biomarkers need to be established for each treatment paradigm and are subsequently validated in clinical studies, ideally based on results from preclinical models. Although no biomarker has been currently validated for a clinical outcome in HD, there are several high potential candidates that are incorporated as exploratory end points in HD clinical studies (https://clinicaltrials.gov: NCT02215616, NCT02197130, and NCT02519036).

TRACK-HD and PREDICT-HD studies involving large groups of HD patients have investigated a wide range of neuroimaging, movement-based, and biofluid-based biomarkers for HD. The results from these studies suggest the potential use of neuroimaging and biofluid-based biochemical markers to measure responses to therapeutic interventions, such as AAV-miRNA gene therapies. For instance, structural MRI can assess reductions in the striatal volume during HD progression. Disease-specific changes of metabolite patterns have been observed using magnetic resonance spectroscopy and positron emission tomography.

Perhaps the most promising is the quantification of biofluid-based disease-associated markers that allow monitoring of HD progression.
following administration of the AAV-miRNA gene therapy.187,188 The soluble mutant HTT concentration in the CSF correlates with disease severity, which suggests that this may become a biomarker to track the effectiveness of HTT lowering.189,190 Major progress has been made in the development of assays that quantify soluble mutant HTT in the CSF.189 It should be noted that the source of mutant HTT in the CSF is not fully understood, and, therefore, its significance as a clinical biomarker for the HTT-lowering efficacy needs robust evaluations. The mutant HTT in the CSF is not linked to functional readouts, ergo, reduced HTT in the CSF doesn’t directly mean functional improvements in patients. Interestingly, the ongoing clinical trial with antisense oligonucleotides recently reported mutant HTT lowering in the CSF in a completed phase I/IIa trial (https://clinicaltrials.gov/: NCT02519036). Next to the mutant HTT, other biomarkers, such as tau, interleukin-6, interleukin-8, and clusterin isolated from CSF and neurofilament light chain (NFL) isolated from plasma were strongly investigated.188–192 A recent study reported that the plasma NFL concentration in HD patients correlated with clinical and magnetic resonance spectroscopy findings in the 3-year international TRACK-HD study. Hence, plasma NFL and mutant HTT in the CSF show the strongest potential so far to become sensitive biomarkers to the AAV-miRNA gene therapy for HD.191

Future Perspectives on HTT-Lowering Gene Therapy for HD
It has been difficult to translate therapeutic HD paradigms into disease-modifying treatments of this devastating disorder. Many studies have failed because of an incomplete preclinical characterization of the mechanism of action and animal studies being poorly designed or underpowered.199 In order to improve the odds of clinical success of gene-silencing therapies for HD, we propose a preclinical framework that addresses, using multiple HD cell and animal models, preclinical measures that are specific for therapies using AAVs as delivery vehicles in order to achieve gene silencing in the corticostriatal circuitry of the brain. The framework includes four main preclinical research phases (Figure 3): (1) a lead selection using in vitro lead candidate selection using either luciferase reporters or iPSC-derived neuronal cultures, rodent and large animal studies addressing major efficacy and safety measures, and good laboratory practice (GLP) toxicology studies. Additionally, tolerability of long-term non-selective HTT lowering in a large animal should be explored.
aggregation) and they should be evaluated in terms of the translational power to clinical trials in HD patients. The overall aim of these studies is to gain sufficient knowledge on the efficacy and safety of an AAV-miRNA gene therapy approach. Once we established consistent miRNA activity across multiple species, we highlighted the importance of careful selection of the AAV serotype together with a route of delivery to achieve safe but efficacious HTT lowering in the striatum and cortex. Importantly, the preclinical studies should strongly focus on the biomarker discovery specific for RNAi therapeutics to facilitate translation into the clinic. In the near future, it will also be important to integrate results from the repeated short-term HTT-lowering clinical studies and the long-term HTT-lowering preclinical studies in large HD animals to better address the consequences of the non-selective HTT-lowering gene therapy in HD patients. Finally, generation of a humanized large animal model that would express both human HTT copies and no endogenous animal HTT would be of great interest to more accurately translate post-treatment preclinical results to humans. To conclude, it is exciting to observe fast preclinical progress of miRNA-based gene therapy approaches and witness AAV-miRNA products currently on the doorstep of clinical trials.

SUPPLEMENTAL INFORMATION
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M.M.E. and P.K. are employees and shareholders at uniQure.

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REFERENCES


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