Utrecht University

Writing assignment

Huntingtin lowering therapies targeting RNA in Huntington's Disease.

Jip Flapper

9129340

Supervisor

prof. dr. Elly Hol

Summary

Huntington's disease is characterized by a progressive deterioration of motor, behavioural and cognitive functioning. The disease is a dominantly autosomal inheritable neurodegenerative disease caused by a genetic mutation in the DNA encoding for the huntingtin protein. An extended CAG repeat in the huntingtin gene causes a gain of toxic properties of the huntingtin protein. Various cellular mechanisms do not function adequately, thereby progressively worsening the performance of multiple cellular pathways. Various animal studies have shown an therapeutic effectiveness of lowering mutant huntingtin levels, resulting in reduced downstream pathogenic effects. Reducing synthesis of mutant huntingtin can be achieved by targeting huntingtin RNA at a post-transcriptional level. Being close to the proximal cause of Huntington's disease, together with the current ongoing human trials, makes RNA targeting currently the most relevant method for therapies applicable in the near future. Huntington's disease patients express the mutant huntingtin as well as the wild-type huntingtin protein. Huntingtin lowering therapies can be designed in two specific ways. Firstly, the allele-selective approach, specifically targeting the mutant huntingtin. The second is in an non-allele specific manner, targeting both the mutant huntingtin and the wild-type huntingtin. The non-allele specific manner raises theoretical risks concerning normal cellular functioning after reduction of wild-type huntingtin levels, but has less limitations for effective targeting of mutant huntingtin RNA. Several reports suggest that lowering both mutant huntingtin and wild-type huntingtin is tolerable up to a certain range, resulting in an overall beneficial effect for Huntington's disease patients. The methods that are efficient in modulating the translation at a post-transcriptional level, either allele selective or non-selective, include antisense oligonucleotides, RNA interference and small molecule modulators. The sequence specific targeting of antisense oligonucleotides allow for specific targeting of mutant huntingtin at pre-RNA level. Repeated intrathecal injection are required for a maintained therapeutic effect. Advantages of antisense oligonucleotides consist of relatively low invasive administration, ability for discontinuation of treatment and broader target range at the pre-RNA level. Disadvantages include need for multiple administrations and inability to spread to deeper brain structures. RNA interference targets RNA at a more downstream location compared to antisense oligonucleotide, reducing the range of possible targets in the processed RNA. RNA interference therapies are most commonly delivered using a viral vector after direct administration into deeper brain structures. Viral vector delivery enables a one time administration of the therapy, but posses a risk due to the inability to discontinue the treatment. The local delivery to the highly effected deeper brain regions could posses a major advantage. The oral availability and widespread action of small molecules make them an attractive approach, however a greater risks of possible off target effect is present. The advanced development and seemingly positive risk-benefit profile indicate that therapies targeting RNA for huntingtin lowering have great potential to be therapeutically beneficial for patients in the near future.

Introduction

Huntington's disease (HD) is a neurodegenerative disease which is dominantly autosomal inheritable. The disease is characterized by a gradual deterioration of motor, behavioural and cognitive functioning, which eventually lead to an early demise[1]. The prevalence of HD varies among geographical regions, varying between 0.40 cases per 100.000 people in Asia to an average of 4 cases per 100.000 people in western Europe countries [2,3]. The progressive manifestation of the disease has a general age of onset between 30 and 40 years of life[4]. There is a considerable variation in how the disease manifests itself. The most common course of the disease is characterized by initial psychiatric symptoms followed by involuntary movements, changes in behaviour and impairment of cognitive functions[5]. The genetic cause of HD is an extended trinucleotide CAG repeat in the DNA coding for the huntingtin (HTT) protein in one of the two copies of the allele (Figure 1)[4]. The age of onset of the disease is inversely correlated to the amount of CAG repeats in the DNA[6]. The CAG repeat length of healthy individuals is below 35, whereas there is a reduced penetrance of the disease if the range of the CAG repeats is between 35 and 39[7]. The majority of patients suffering from HD have CAG repeat length above 40.

The extended CAG repeat results in the production of the mutant huntingtin (mHTT) protein. The huntingtin gene (HTT) contains the CAG repeat on exon 1 at the 5' end, which translates into a corresponding polyglutamine amino acid tract in the HTT protein (Figure 1)[4]. HTT is a protein expressed ubiquitously throughout the human body, which has been shown to be crucial for embryonic development[8]. HTT has shown to contribute to neuronal survival through anti-apoptotic properties and protection from excitotoxity[9]. The protein enables correct functioning of important mechanisms which are essential in a healthy brain. HTT was found to influence transcription of various neuronal genes, as well as regulation of axonal trafficking, vesicle transport and synaptic transmission[10–14].
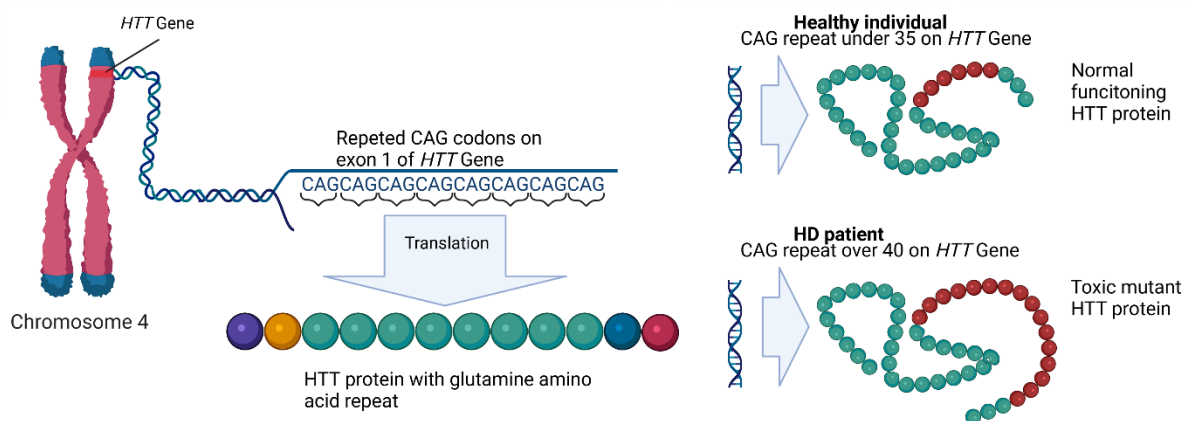

Figure 1 – HTT gene on chromosome 4 with the CAG repeat expressing the HTT protein. Healthy individuals express the HTT protein with up to 35 glutamine repeats in the protein. HD patients express the toxic mHTT protein with an extended glutamine repeat. Image created with Biorender.com

Expression of the extended CAG repeats in the HTT gene can lead to multiple toxic variants which are involved in various degrees during the complex pathogenesis of HD (Figure 2). Pathological changes arise rather selective in the central nervous system irrespectively of ubiquitous mHTT expression[15]. The abnormal functionality of mHTT and its aggregation in the central nervous system is the main pathological characteristic of the disease. The mHTT protein tends to misfold and aggregate due to the extended polyglutamine amino acid tract[16]. The full-length mHTT protein gains toxic properties and is unable to perform several functions adequately, thereby progressively

worsening the functioning of multiple cellular pathways[5]. The mHTT dysregulates transcription of various important neuronal genes, reduces axonal trafficking, impairs vesicle transport, causes mitochondrial dysfunction and contributes to abnormal synaptic transmission[11–14,17]. These dysregulations cause several implications among different cell types, causing cellular stress and activation of cellular death pathways.
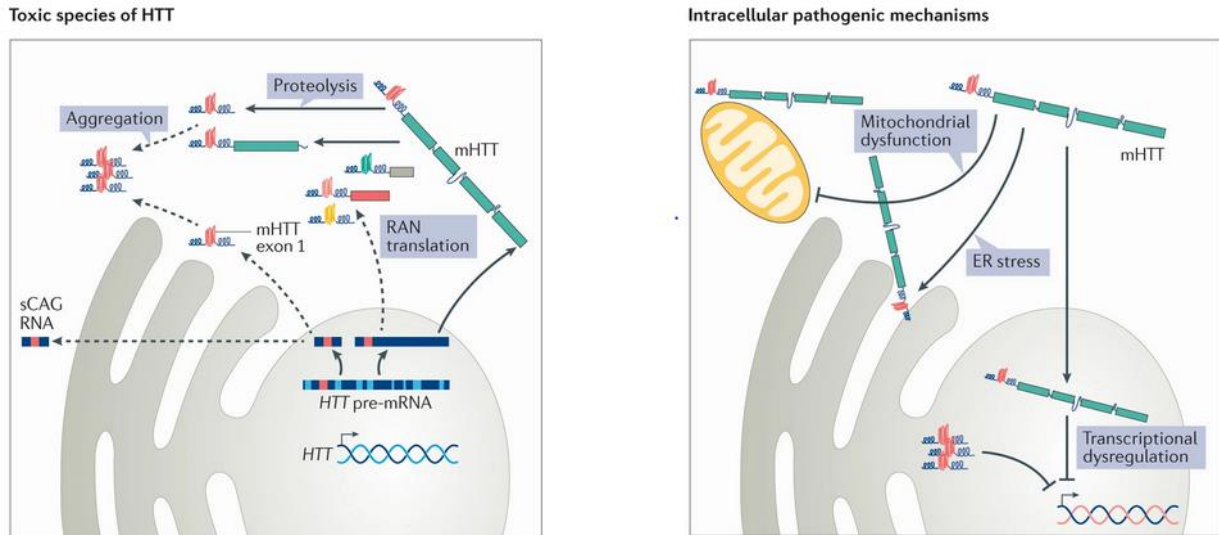

Figure 2 – Toxic variants generated by the mutation in the HTT gene contribute to disease pathology in various degrees. Full-length mHTT, fragments of the protein, small CAG RNA and RAN translated proteins gain toxic functions or aggregate inside the cell. mHTT causes various pathological mechanisms inside the cell including mitochondrial dysfunction, transcriptional dysfunction and stress in the endoplasmic reticulum. Image adapted from "Therapeutic approaches to Huntington disease: from the bench to the clinic" Caron et al., 2018, Nat Rev Drug Discovery 17(10):729-750. doi:10.1038/NRD.2018.133 [5]

Aggregation of smaller proteolytically cleaved N-terminal polyglutamine fragments of mHTT are considered to also play a role in the pathogenesis of HD[18,19]. During the process of mHTT synthesis various other toxic variants can arise. After the *HTT* gene is transcribed from the DNA, the pre-mRNA of the mHTT is located inside the nucleus. The extended CAG repeats on exon 1 can trigger aberrant splicing, causing production of pathogenic exon 1 HTT RNA[20]. This exon 1 HTT RNA includes the CAG repeats at the N-terminus. The exon 1 HTT RNA can be translated to pathogenic polyglutamine protein fragments, which attribute to aggregation in a similar manner as proteolytically cleaved N-terminal polyglutamine fragments of mHTT. In recent years there has been emerging evidence that the CAG repeated RNA itself is linked to cellular defunction in HD through RNA-mediated molecular mechanisms[16,21]. The CAG repeated RNA can gain toxic functions due to aberrant attachments to proteins or other RNA molecules, causing deregulation in RNA localization, splicing, microRNA functioning, mitochondrial RNA or translation. Another potential toxic variant that has been elucidated in recent years are repeat-associated non-ATG (RAN) proteins. RAN proteins are created due to CAG repeat expansion mutations, which can initiate the production of proteins in a start codon independent manner[22]. RAN proteins aggregation associated with HTT were found in human HD brain autopsies, however their role and toxicity in HD pathogenesis remains unclear[23–25]. The different toxic variants may contribute to the pathogenesis in various ways, resulting in the progressive deterioration of neuronal function. Dysfunction of various cells and their impaired interaction can be seen as a result of this progressive deterioration (Figure 3). Impaired synaptic glutamate uptake and disrupted K+ homeostasis can be seen in astrocytes[26,27]. Cortical neurons experience reduced trophic factor molecule expression and impaired transport of these factors[10,28]. Medium spiny neurons experience loss of trophic factors, resulting in reduced growth and survival.

Additionally, medium spiny neurons experience further excitotoxity due to Ca^{2+} dysregulation and aberrant synaptic signalling[5,26]. Activated microglia can cause neuroinflammation through aberrant immune activation[29]. Cellular deterioration eventually manifests itself as the clinical motor, behavioural and cognitive changes.
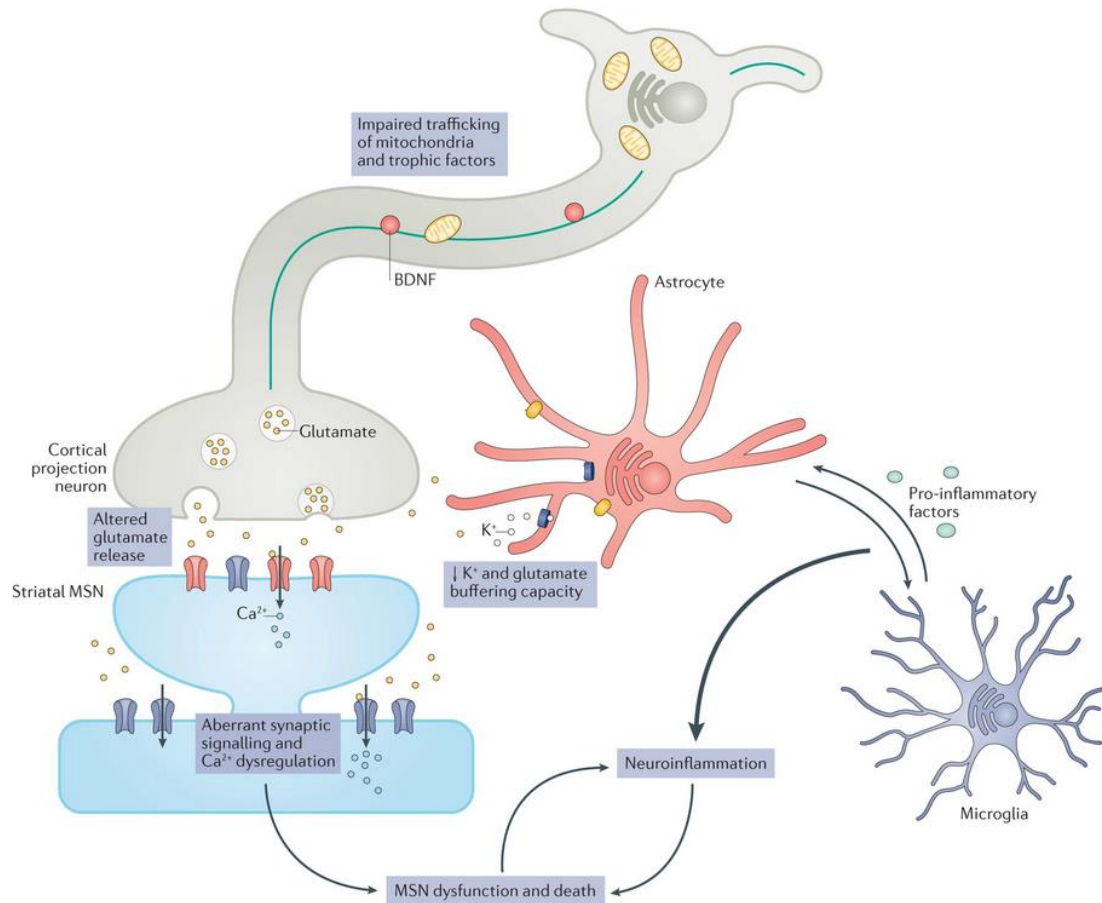

Figure 3 – Various cells and their processes are effected by the intracellular disease pathology. Astrocytes display impaired synaptic glutamate uptake and disrupted K^+ homeostasis. Trophic factor expression and transport is reduced in cortical neurons, contributing to the disruption of glutamate homeostasis. Reduced growth and survival can be seen in medium spiny neurons, due to loss of trophic factors and excitotoxicity. Microglia contribute to Increased neuroinflammation due to dysregulated immune activation. Image adapted form "Therapeutic approaches to Huntington disease: from the bench to the clinic" Caron et al., 2018, Nat Rev Drug Discovery 17(10):729-750. doi:10.1038/NRD.2018.133 [5]

The main focus of current therapies being developed is lowering the mHTT levels, thereby eliminating downstream pathogenic effects. Animal studies have shown that lowering mHTT levels can improve or even partially reverse disease phenotype in HD[30,31]. Reducing the synthesis of mHTT can be achieved by targeting RNA, through inhibiting post-translational processes or inhibiting translation. Targeting the most causative cause of HD would most likely be the most effective therapy. Not only by means of lowering mHTT levels, but also in diminishing alternative pathological mechanisms caused by toxic variants. Even though targeting the CAG-expended *HTT* gene in the DNA is the most proximal target for mHTT lowering therapies, genome editing techniques still require additional research and pre-clinical studies before they can be safely applicable for human clinical trails. Several therapies targeting RNA at a post-transcriptional level have already gone into human clinical trials. These ongoing human trials and the close proximity to the cause of HD, make RNA

targeting currently the most relevant method for HD therapies. This review will focus on HTT lowering therapies targeting RNA in HD.

The HTT protein is encoded on two different copies in the DNA, on one allele from the father and one from the mother. Almost all HD patients have the causative extended CAG tract mutation on only one of the two alleles. One allele expresses normal wild-type HTT, while the other expresses the HD associated mHTT. Therapies targeted at lowering HTT levels can be designed in two specific ways. The first one being HTT lowering treatment in a allele-selective manner, specifically targeting the mHTT. The second is in an non-allele specific manner, targeting both the mHTT and the wild-type HTT. The toxic gain of function of mHTT makes it a crucial target for clinically efficiency[32]. The allele specific manner has the advantage of only affecting pathogenic mHTT, without affecting the natural cellular functioning of wild-type HTT. It has been shown that wild-type HTT is crucial for embryonic development in murine models[8]. The non-allele specific manner raises theoretical risks concerning normal cellular functioning after reduction of wild-type HTT levels. Considering the progressive nature of HD, therapies will likely need to be implemented early in the adult life, before manifestation, and continue throughout life. The risks of long term wild-type HTT reduction are unknown and could limit clinical feasibility of non-allele specific targeting of HTT. However, there have been several reports were individuals heterozygous for the null variant of HTT show no pathological phenotype, suggesting that wild-type HTT levels reduced to 50% are still sufficient for normal development and cellular functioning[33–36]. This suggest that lowering both mHTT and wild-type HTT could have an overall beneficial effect for HD patients. The methods that are efficient in modulating the translation at a post-transcriptional level include antisense oligonucleotides (ASOs), RNA interference and small molecule modulators. These methods lead to the reduction of mHTT protein through translational suppression, enhanced degradation or initiating cleavage of mHTT RNA[32].

ASO therapies

ASO approaches use synthetic short single stranded DNA molecules which bind predominantly to pre-mRNA through Watson-Crick binding[37]. Chemical modification of ASOs allow for longer half-life, improved duration of action and better penetrance into target cells. Depending on design, the ASOs can have various modes of action (Figure 4). ASOs can bind to pre-RNA, causing degradation through endogenous enzymes[38]. ASOs bound to mRNA can stall translational machinery causing translational arrest[37]. Lastly, ASOs can alternate pre-RNA splicing. Masking specific splicing sequences can result in the expression of non-toxic variations of the protein. ASOs have a limited ability to cross the blood-brain barrier, thus direct delivery into the central nervous system is preferable[39]. Lower doses are needed for effective concentrations, resulting in a lower risk for toxicity. Rodent models have shown an even distribution of ASOs throughout the central nervous system, however larger brain studies show a lower concentration gradient of ASOs in deeper brain structures after delivery into the central nervous system[38].
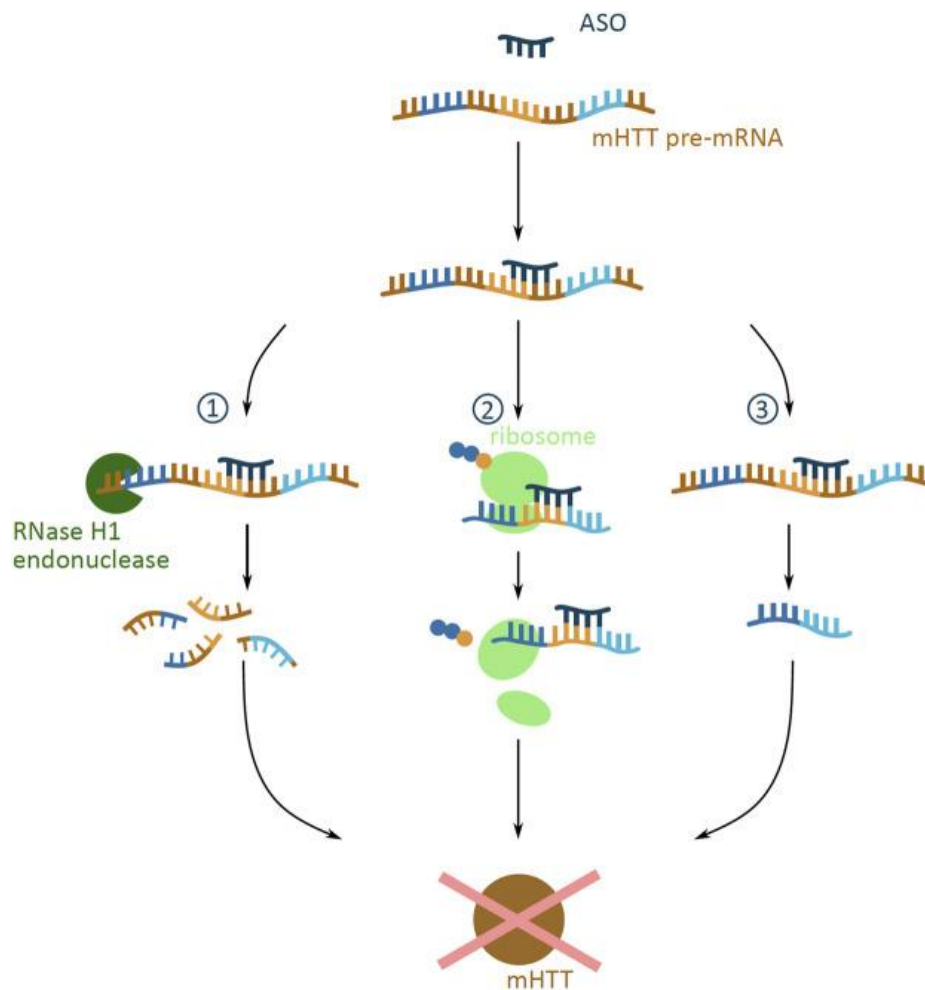
*Figure 4 – Various mechanisms of action of ASOs. ASOs can inhibit the production of mHTT protein after binding to the HTT pre-RNA inside the nucleus. **1**. RNase H1 can be recruited after binding and initiate the endogenous degradation mechanism. **2**. Bound ASOs can cause translational arrest. **3**. Bound ASOs can mask specific splicing sequences, thereby modulating RNA splicing. Image from "Molecular Strategies to Target Protein Aggregation in Huntington's Disease." Jarosińska, O. D. & Rüdiger, S., 2021, Frontiers in molecular biosciences /doi.org/10.3389/fmolb.2021.769184* [40]

The sequence specific targeting of ASOs allow for highly specific modulation of mHTT at a post-transcriptional level. Unmodified ASOs are vulnerable to nuclease cleavage, resulting in rapid degradation *in vivo*[39]. In the past decades, advances in medical chemistry enabled chemical modifications for ASOs which allowed for a better applicability in a clinical setting. ASOs used for reduction of huntingtin rely on the antisense mechanism where the RNA is targeted and degraded. In order to make ASOs appropriate for clinical applications they need high nuclease resistance, high binding affinity and activation of RNase-H[41]. RNase-H mediates the cleavage of the target RNA, resulting in the degradation of the target RNA. ASOs do not only target the RNA in the cytosol, but can also target unprocessed pre-RNA inside the nucleus[42]. The ability to target pre-sliced RNA allows for targeting introns as well as exons of the RNA, increasing the amount of possible targeting sides. High activity and species selective ASOs have been identified after full-length mouse and human HTT screening[38]. The ability of the ASO the suppress human mHTT RNA and mHTT protein was shown in the BACHD and YAC128 mouse models in a dose dependant manner. The BACHD mouse model, which expresses full-length human mHTT with a CAG repeat length of 97, and the YAC128 mouse model, which expresses human mHTT with a CAG repeat of 128, both develop progressive cognitive and motor impairments highly similar to HD neuropathology[43,44]. Broad distribution of the ASO was achieved throughout the brain of the mouse models and suppression of mHTT RNA levels lasted 2 or

3 months[38]. Suppression of the human mHTT in both mouse models let to phenotypical reversal in the disease models. In the mouse models only mutant HTT was targeted using this ASO, because wild-type endogenous mouse HTT has a different targeting sequence[38]. However if applied in humans both wild-type and mutant HTT would be targeted by this ASO. In order to assay the safety and effectivity of lowering both wild-type and mutant HTT another ASO was used which targets both mutant human as well as wild-type mouse HTT. The ASO applied to the YAC128 and BACHD mouse models showed an effective lowering of both endogenous wild-type HTT and mutant human HTT[38]. Suppression of mHTT let to disease reversal in both mouse models, improvements of disease phenotype were unaffected by reduction of wild-type HTT levels. There was no evidence of toxicity or aberrant motor functioning after suppression of wild-type HTT. Most interestingly, partial disease reversal was shown to persist for at least 4 months after HTT RNA and protein levels had returned to basal level, indicating a benefit long after HTT suppression has ended. The brain of rodent models is substantially different from the human brain anatomically and in size. In order to better asses the distribution for human application, the ASO was injected intrathecally in nonhuman primates[38]. The ASOs were distributed rather broadly, showing reduction of HTT various regions of the brain effected by HD up to 2 months after injection. The deeper brain structure were effected in a lesser extend, indicating a higher concentration of ASOs in cortical regions.

Despite these encouraging results, concerns remain about the effect of long-term wild-type HTT suppression on the adult nervous system. The effect on normal functioning of HTT after long term partial suppression is unknown. There is evidence that complete loss of wild-type HTT has detrimental consequences. HTT is known to be crucial for embryogenesis, furthermore has it been shown that complete inactivation of HTT in adult mice leads to neurodegeneration[8,45]. The role of wild-type HTT in transcription, exonal trafficking, vesicle transport and synaptic transmissions indicates that HTT has an essential role in the postnatal brain. The allele specific targeting of mHTT might be advantageous in circumventing possible risks associated with reduced wild-type HTT. Allele selective targeting of mHTT may be possible through targeting extended the CAG-tract directly or targeting genetic single-nucleotide polymorphisms (SNPs). Various ASOs have shown to inhibit mHTT selectively by targeting the extended CAG tract[46,47]. These studies achieved a significant allele selectivity for suppression of mHTT in patient derived fibroblasts and the YAC128 mouse model. Achieved selectivity was not higher than 4 to 8 fold compared to wild type HTT[46,47]. However the highest specificity was achieved in CAG repeats which are considerable longer (> 69 CAG repeats) than most HD patients posses (40-45 CAG repeats)[7]. Indicating the possibility of diminished selectivity at more commonly observed CAG repeat lengths in HD patients. Furthermore, the expanded CAG tract can be seen in numerous other transcripts, their non-specific down-regulation could have deleterious effects. Another strategy for allele specific targeting of mHTT is the targeting of SNPs associated with the extended CAG tract. A SNP is a single nucleotide variation in the genetic sequence. ASOs have the ability to bind to different target RNAs based on different SNPs[48]. Most SNPs in the human DNA have little to no influence on cellular function, however some SNPs can be associated with certain diseases. These SNPs can be useful for diagnostic or specific targeting purposes. Numerous SNPs have been identified in the *HTT* gene region which are associated with the extended CAG tract[49]. Meaning that these SNPs can be found on the allele coding for mHTT but not on the other copy of the allele coding for wild-type HTT. A disadvantage of allele specific targeting through SNPs is that not every HD patient posses the same disease associated SNP. Multiple SNPs would have to be identified and targeted for treatment applicable to all HD patients globally. The most common SNP associated with the extended CAG tract identified occurs in roughly 50% of the HD patients[49]. Targeting a panel of 3 to 5 different SNPs associated with the extended CAG tract could provide treatment options for 80%-90% of HD patients around the world[50]. Several

ASOs were developed targeting SNPs in the human *HTT* gene that selectively silenced mHTT expression[51]. Four potent ASOs were selected from the initial screening and tested in YAC18 and BACHD mouse models. YAC18 mouse models expresses human wild-type HTT instead of murine wild-type HTT, the human HTT is functional in the murine background and compensates for loss of murine HTT[52]. The ASOs suppressed the allele specific human mHTT in the BACHD mouse model, but did not supress the human wild-type HTT in the YAC18 mouse model[51]. Suppression between 40% and 60% of mHTT could be observed, with negligible suppression of wild-type HTT. In order to better evaluate the effect of the ASOs, the Hu97//18 mouse model was created. The Hu97//18 mouse model expresses full-length human mHTT (97 CAG repeats) with HD associated SNPs, as well as full-length human wild-type HTT without HD associated SNPs[53]. This model allows for better screening and evaluation of ASOs as both wild-type and mutant HTT are expressed in the same brains and cells. Screening in Hu97//18 mice revealed that some of the previously identified ASOs were less selective as previously thought[54]. Through screening of Hu97//18 several promising ASOs were found, which showed therapeutic efficacy in the model. Distribution and tolerability of the ASOs was evaluated in nonhuman primates. The gradient, with a low concentration of ASOS in deeper brain structures, found after injection in nonhuman primates, necessities the need for ASOs with a large therapeutic window of safe and efficacious doses. Four ASOs were identified based on efficacy and tolerability, which show promise for therapeutic application for allele specific targeting of mHTT.

Both selective and non-selective reduction of mHTT through ASOs have shown potential in various animal models. Both approaches have their own benefits and risks. One advantage of non-selective HTT silencing is the potential for a single therapeutic for the entire HD patient population compared to allele specific SNP based targeting. Additionally, there is a broader target rage which is not limited to SNP associated sequences. The chance of finding a highly potent and specific target increases because there is greater region available to target. The disadvantage of non-selective HTT silencing is the possible dysfunction due to the suppression of wild-type HTT. Even though tolerability has been reported in various animal models for reduced wild-type HTT, the long-term effects on humans are still unknown. The crucial function of wild-type HTT in brain cells requires extreme caution when intervening. Allele specific targeting of mHTT through SNPs avoids altering the levels of wild-type HTT, while simultaneously suppressing mHTT. Thereby, eliminating any possible negative effects of long-term reduction of wild-type HTT.

Both allele selective and non-selective approaches are currently being pursuit in clinical trials. A non-selective ASO has firstly been developed by Ionis Pharmaceuticals and licensed to Hoffman-La Roche for further development and commercialization. Tominersen (also known as ISIS 443139, RG6042, RO7234292 or IONIS-HTTRx) is an ASO designed to bind to HTT RNA, initiating RNase mediated degradation, resulting in reduced levels of HTT protein[55]. A Phase 1/2a clinical trial was set up in 2015 to evaluate safety of the drug (ClinicalTrials.gov number, NCT02519036). Adults with early HD received Tominersen or a placebo in increasing doses. The study was completed in 2017 and showed that intrathecal administration of the drug was not accompanied by serious adverse events. Secondary endpoints showed a dose dependent reduction in mHTT in the cerebrospinal fluid (CSF) of the participants. Preclinical studies indicate that the reduction of mHTT in the CSF is reflecting the mHTT concentration in the central nervous system[55]. Patients who completed the trial could participate in an extension of the Phase 1/2a clinical trial (ClinicalTrials.gov number, NCT03342053). The extended trial, which examined the safety, tolerability, pharmacokinetics and pharmacodynamics of the drug for a longer period, has also been completed. In 2019 a Phase 3 study enrolled 791 participants (ClinicalTrials.gov number, NCT03761849). The study was designed to primarily evaluate the efficacy of the drug. The trial did not focus on mHTT reduction, but rather improvements of clinical manifestations of the disease defined as functional ability, motor function

and cognition. Roche announced in March 2021 that it was stopping dosing in the Phase 3 study based on a recommendation from a independent data monitoring committee[56]. Analysis from the committee found that patients overall received no benefit from the treatment. Roche announced in January 2022 that finding from post hoc analysis of the data from the Phase 3 trial suggested that younger patients with less disease burden might benefit from the treatment[56]. The company is initiating a programme where the focus is more on this subpopulation of HD patients using the treatment.

A selective approach has been developed by Wave Life Science. The ASOs WVE-120101 and WVE-120102 are designed to specifically target the extended CAG tract associated SNP1(rs362307) and SNP2 (rs362331). These ASOs would selectively suppress mHTT while wild-type HTT levels remain unaffected. Phase 1b/2a clinical trials were initiated in 2020 for evaluation of safety and tolerability of the ASOs (ClinicalTrials.gov numbers, NCT03225833, NCT04617847, NCT03225846 and NCT04617860). Wave Life Science reported in 2021 that the trials would be discontinued[57]. Even though adverse events were mostly mild to moderate across the trials, the data showed no evidence of dose-response across the dose levels tested. No significant change in mHTT could be seen in the CSF of the participants. The company reported that in the open label extension study of WVE-120102 some participants had a mHTT reduction of 20% without any observable correlation with wild-type HTT change, suggesting that allele selectivity had been achieved. Wave Life Science continues the development of allele selective mHTT reduction with another ASO. The WVE-003 targets another SNP associated with the extend CAG tract and has novel chemical modification compared to WVE-120101 and WVE-120102. A phase 1/2a clinical trial evaluating safety and tolerability for WVE-003 has started in 2021 and is currently ongoing (ClinicalTrials.gov numbers, NCT05032196).

Both allele selective and non-selective approaches hold great promise for future treatment of HD. Even though both approaches have not achieved their ultimate goal of efficacy in the clinical trails previously discussed, their use was considerable safe and well tolerable in humans. Further dosing studies and chemical optimization need to be completed in order to find the ideal and most beneficial applicability of the ASOs for human trials.

RNA interference therapies

The RNA interference approach is based on using endogenous RNA interfering machinery for post-transcriptional sequence-specific gene silencing. During the endogenous interfering pathway a double stranded RNA (dsRNA) is processed into a functional small single stranded RNA (siRNA) , which acts as a guide strand[58]. The siRNA associates with several proteins, forming the RNA-induced silencing complex (RISC). The siRNA guides the complex to a complementary target RNA and induces degradation of the target RNA. This pathway can be manipulated by using specifically designed artificial siRNA to target and degrade mHTT RNA (Figure 5). The artificial siRNA can be introduced directly into the cell as a single stranded siRNA molecule or encoded into a viral vector. Direct addition of siRNA is has two major disadvantages. The inability of crossing the blood-brain barrier and limited distribution across central nervous cells[59]. Carrier formulations or chemical modifications need to be utilized for improved cell entry and distribution. The short-term effect would require repeated administration directly into the central nervous system, which can be quite invasive. More common is the use of viral vectors for delivery (Figure 5). The vector can be designed to express dsRNA short hairpin RNA (shRNA) or artificial micro RNA (miRNA)[60]. The expressed dsRNA is then further processed to form the single strand siRNA guide strand for the RISC complex. Currently used vectors are adeno-associated virus (AAV) vector or lentivirus (LV) vector, both cannot replicate in the host and are non-pathogenic[32]. AAV vectors will not integrate into the host genome, their expression

of the interference RNA will dilute over time due to repeated rounds of cell replication[61]. This dilution is minimal in brain cells , due to vector optimization and limited replication of brain cell[62]. The expression of interference RNA will remain relatively stable for LV, because LV do integrate into the host genome. However, insertion into the host genome has additional risk of insertional mutagenesis. Viral vector based delivery offers a longer lasting expression of the interference RNA molecules, thereby limiting the repetitive invasive delivery into de central nervous system.
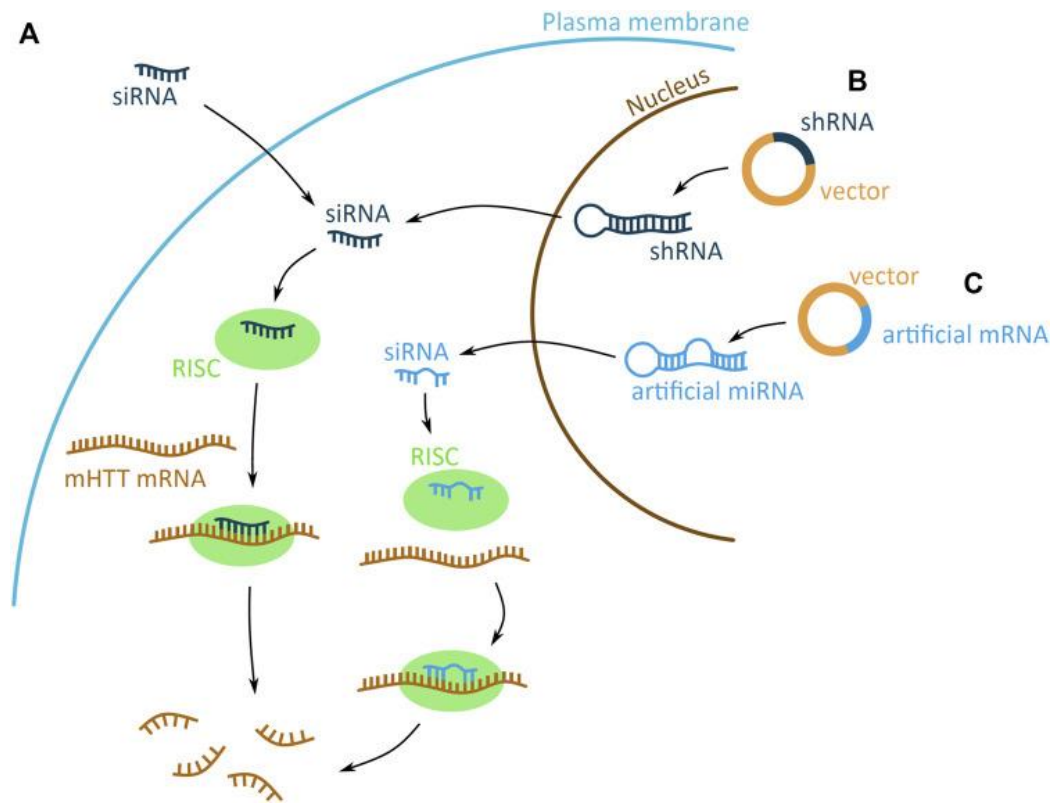

*Figure 5 – Various application of endogenous RNA interference machinery. **A**. Artificially designed siRNA can be added directly to the cell. Then forming the RISC complex and inducing degradation of the targeted RNA. **B** and **C**. Artificially designed shRNA or miRNA can be added to the cell using a viral vector. The vector expresses the shRNA or miRNA, which is further processed into siRNA. Followed by the formation of the endogenous RNA degradation complex and degradation of the target RNA. Image from "Molecular Strategies to Target Protein Aggregation in Huntington's Disease." Jarosińska, O. D. & Rüdiger, S., 2021, Frontiers in molecular biosciences /doi.org/10.3389/fmolb.2021.769184 [40]*

The RNA interference approach targets the mHTT RNA more downstream compared to ASOs. The processed RNA in the cytosol is targeted by the RNA interference machinery, instead of pre-RNA in the nucleus[32]. Artificially designed siRNA can be used to manipulated the endogenous pathway for short-term lowering of mHTT. Direct addition of artificially designed siRNA needs no further processing, the interference machinery is promptly activated after cell entry. Protein levels of mHTT have successfully been lowered using siRNA in several mouse models, resulting in reduced neuronal pathology and improved motor function[63,64]. Nonhuman primate studies showed a considerate suppression of HTT in the deeper brain structures after administration of chemically modified siRNA[65,66]. The inability of siRNA to cross the blood-brain barrier and limited brain distribution capacities necessitate direct injection into deeper brain regions[59]. The nonhuman primate studies indicate that a repeated administration of the siRNA to the deeper brain regions is needed for a therapeutic effect[65,66]. The ability the stop the treatment at any time is an advantage if need for discontinuation arises due to safety concerns. However, repeated administration into the deeper brain structures of HD patients presents a high life long burden.

Long-term HTT lowering after one time delivery would have major benefits for HD patients. Viral delivery of siRNA precursors, such as shRNA and miRNA, can realize long-term huntingtin lowering. Artificial shRNA and miRNA therapeutics operate similar to artificially designed siRNA, mature RNA is targeted sequence specifically and degraded by endogenous RNA interference machinery[32]. shRNA and miRNA differ from siRNA because they first need to be processed into the guide siRNA by endogenous cellular processes[58]. shRNA and miRNA are both expressed as sense and anti-sense sequences connected by a loop of unpaired nucleotides[67]. They will both eventually express the same functional siRNA, however their cellular processing is different. AAV or LV vectors are most common delivery methods for shRNA or miRNA. In early development of RNA interference treatments, HTT RNA and protein expression was successfully reduced in human cell lines resulting in improved cellular survival[68]. Numerous animal studies have been performed to evaluate the safety and efficacy of viral delivery of shRNA or miRNA (Table 1). Early studies in mouse models showed encouraging results. The N171-82Q mouse model, encoding a N-terminal human mHTT with 82 CAG repeats, showed reduced mHTT RNA and improved motor functions[69,70]. The R6/1 mouse model, encoding human mHTT with 150 CAG repeats, showed similar improvements and delayed onset of HD[70,71]. These studies showed a promising result in pre-symptomatic mouse models, the shRNA used were administered into the striatum before manifestation of the disease. The HD190QG mouse model expresses a 190 CAG repeat human mHTT, the mice show less viability and can be used as a model for when HD pathology has manifested[72]. A reduction of mHTT was achieved in the HD190QG mouse model using shRNA, resulting in improved HD associated abnormalities. Yet reversal of disease phenotype could not be observed. This indicates the importance of the timing of HTT lowering treatments with regards to degree of disease manifestation. It is unclear if complete reversal of symptoms is possible after disease manifestation.

Several other studies showed that the reduction of human HTT was possible in rodent models using different techniques. It was shown that LV vector delivery was also a plausible candidate for successful delivery of shRNA[73]. Reduced mHTT and improved HD pathology were observed after LV shRNA delivery. Other research showed that certain mHTT lowering shRNA could induce toxicity in the mouse striatum[74]. Notably, it was shown that neurotoxicity could be diminished, without effecting HTT lowering capacities, if the toxic shRNA variants were placed into artificial miRNA expression systems. Indicating that miRNA approaches may provide a more biological applicable tool when interference RNA structures are expressed in the brain. Most of the aforementioned studies only lowered the human mHTT levels in transgenic mouse models, not effecting the endogenous wild-type HTT. Application of the shRNA/miRNA in humans would lower both mutant and wild-type HTT. Various studies administered miRNA/shRNA into the striatum of rodent models, targeting both human mHTT and endogenous wild-type HTT[73,75,76]. Human mHTT as well as endogenous HTT was successfully reduced in the studies, accompanied by improvements of HD associated behavioural abnormalities and pathologies. Furthermore, could no toxicity be observed as a consequence of reduced endogenous wild-type HTT. The reduction of wild-type HTT by shRNA and miRNA has also been shown to be safe and non toxic in nonhuman primates[77,78]. A 45% reduction of wild-type HTT was achieved up to 6 weeks after miRNA administration into the striatum[77]. The reduction did not lead to any observable neuropathology or behavioural symptoms. Prolonged reduction up to 6 months after administration of a shRNA in another nonhuman primate study showed similar safety and tolerability[78]. Despite these results, concerns remain about of non-selective HTT lowering applied in humans. Various efforts have gone into developing RNA interference methods which can reduce mHTT in a allele selective manner[79–81]. *In vitro* experiments have shown significant allele selectivity of shRNA/miRNA targeting SNPs, resulting in lowered mHTT levels. However, these results

have not been adequately translated into *in vivo* experiments in rodent models. Difficulties could be seen *in vivo* in effective mHTT reduction while maintaining sufficient allele selectivity.

The anatomical difference between rodent brains and human brains is rather large. Additional research is required to better asses distribution and efficacy of viral vectors expressing interference RNA delivered into larger brains. A HD sheep model, expressing full length human mHTT with 73 CAG repeats, was used to better asses distribution and efficacy in large animal brains[82]. Effective human mHTT silencing was achieved and sustained up to 6 months after injection. Vector concentration was mainly focussed near the injection side, indicating higher concentrations of miRNA in deeper brain structures. Injection of a vector expressing miRNA into a HD minipig model, expressing human mHTT with 124 CAG repeats, showed similar results[83]. Long term miRNA expression accompanied by reduction of human mHTT RNA and protein was achieved. These large animal brain studies support the notion that viral vector delivery of RNA interference is applicable for HD patients.

Non-selective					
Animal model	RNAi	Vector	location	Research outcomes	Ref.
N171-82Q Mouse	shRNA	AAV1	striatum	A reduction of human mHTT RNA and improvement of behavioural deficits was shown in the mouse model.	69
R6/1 Mouse	shRNA	AAV5	Striatum	A reduction of human mHTT RNA and protein was shown in the mouse model. Additionally a delayed onset of phenotypical pathology was observed.	71
HD190QG Mouse	shRNA	AAV5	Striatum	A reduction of human mHTT showed to improve HD-associated pathological abnormalities for the post-symptomatic HD mouse model.	72
CAG140 Mouse	shRNA miRNA	AAV1	Striatum	Similar human mHTT reduction was seen between shRNA and miRNA, however less toxicity was observed when the construct was placed into artificial miRNA expression systems.	74
N171-82Q Mouse	miRNA	AAV1	Striatum	Non-allele specific suppression of human mHTT and wild-type mouse HTT improve HD-related behavioural abnormalities in the HD mouse model.	75
N171-82Q Rat	shRNA	LV	Striatum	Lentiviral administration post-symptomatic is efficacious and reduces HD-pathology. Additionally, non-allele selectivity did not result in observed toxicity.	73
YAC128 Mouse	miRNA	AAV1	Striatum	A reduction of human mHTT and wild-type HTT was shown in the mouse model, corresponding with improvements behavioural deficits. Additionally, non-allele selectivity did not result in observed toxicity.	76
Q175 Mouse	miRNA	AAV5	Striatum	Long term efficacy was observed, HTT protein lowering was observed up to 12 months after treatment.	84
Selective					
BACHD Mouse N171-82Q Rat	shRNA	LV	Striatum	Allele selective silencing of mHTT was achieved *in vitro* by targeting several SNPs associated with HD. *In Vivo* experiments showed potential for allele selectivity, but no definite evidence.	81
Hu128/21	miRNA	AAV5	Striatum	Allele selective silencing of mHTT was achieved *in*	79,80

Mouse Acute LV HD model Rat				*vitro* by targeting several SNPs associated with HD, but translated poorly to *in vivo* applications.	

Animal model	RNAi	Vector	location	Research outcomes	Ref.
Wild-type Nonhuman primate	miRNA	AAV1	Striatum	A reduction of 45% of HTT was well tolerable in non human primates. No induced motor deficits, neuronal degradation, astrogliosis or immune response was observed 6 weeks after injection.	[77]
Wild-type Nonhuman primate	shRNA	AAV2	Striatum	A wide spread reduction of HTT was observed 6 months post injection, furthermore no additional safety concerns were found.	[78]
HD sheep	miRNA	AAV9	Striatum	Effective and sustained silencing of human mHTT was achieved in the large-animal brain.	[82]
HD minipig	miRNA	AAV5	Striatum	Effective and sustained silencing of human mHTT was achieved in the large-animal brain.	[83]

Table 1 – Various animal models used for viral delivery of shRNA or miRNA

Two clinical trials have been approved for RNA interference therapy for HD patients. UniQure has developed AMT-130, a AAV5 vector encoding miRNA which targets HTT. Safety and proof of concept are the main endpoints of the first in human clinical trial (ClinicalTrials.gov number, NCT04120493). AMT-130 will be administered into the caudate and striatum. The trial was initiated in 2020 and is currently still ongoing, UniQure reported in December 2021 that the first two patients dosed with low doses of AMT-130 showed no significant safety issues at the end of the first year after administration[85]. Another safety and proof of concept clinical trial had been approved in 2021. VY-HTT01 has been developed by Voyager Therapeutics, the drug is a AAV1 vector encoding miRNA which targets HTT (ClinicalTrials.gov number, NCT04885114). Later in 2021 Voyager announced that the trial was discontinued. The company announced that it will shift its focus to a novel proprietary AAV capsid, initiating a programme where the RNA interference vector may be administered intravenously[86].

Small molecule therapies

Challenges remain for delivery of ASOs and RNA interference therapeutics into the brain. Both approaches require direct administration into the central nervous system, due to limited penetrance of the blood-brain barrier. This can result in irregular distribution through the central nervous system and inconsistent HTT lowering. Additionally, direct administration into the central nervous system can be quite invasive for HD patients. Orally available small molecules which can reduce HTT expression throughout the brain are highly desirable. Although there is an increased risks of off-target effects, optimizing small molecules could provide a feasible HTT lowering therapy. After screening diverse molecules in a HTT protein assay using HD patient derived fibroblast, several compounds were identified which lower HTT levels[87]. The small molecules modulate the splicing of HTT pre-RNA by inducing the inclusion of a pseudo exon, which contains a premature termination codon. Inclusion of the premature termination codon into the RNA results in the degradation of HTT

RNA. The small molecules was shown to be orally available and exert their effect evenly distributed throughout the brain and the periphery in BACHD and Hu97/18 mouse model. A clinical trial by Novartis Pharmaceuticals has been approved using a previously described small molecule (ClinicalTrials.gov number, NCT05111249). LMI070 (also known as Branaplam) has been approved for a dose finding study focussing on the safety, tolerability and efficacy of HTT lowering. The trial was initiated in 2021 and is currently ongoing.

Discussion

The mutation leading to the production of the aberrant mHTT protein arises at the same location in the DNA for all HD patients[4]. The highly specified and well known location of the mutation enables opportunities for highly specific gene silencing therapies. The RNA targeting therapies discussed show great promise in targeting the extended CAG tract mutation, resulting in mHTT reduction. The concerns about lowering wild-type HTT remain for all types of RNA targeting therapies. Currently the most advanced therapies developed target mHTT in a non-selective manner, lowering both wild-type and mHTT. The non-selective approach offers some advantages. A single treatment is available for the entire HD population, compared to patient specific SNP targeting necessary for allele selective targeting. Additionally, the degree of mHTT lowering seems to be the driver for therapeutic benefit. Comparison of a selective ASO and non-selective ASOs in a mouse model indicated that therapeutic effects were the highest for the ASO with the highest mHTT reduction, independent of wild-type reduction[88]. The main concern for non-selective treatment is possible safety risks in lowering endogenous HTT. The crucial role of HTT in development and neuronal cell functions raises concerns for long term reduction[8,45]. Evidence from specific individual cases of heterozygous null variants of HTT and several animal studies indicate that a reduction of 50% wild-type is well tolerable[33–36,38,73,75–78]. Tolerability of wild-type HTT reduction is supported by safety and tolerability of non-selective ASOs in human clinical trials. The efficacy of non-selective approaches in reducing HTT has not been translated into therapeutic benefit in clinical trials yet. Nevertheless, the non-selective approach seems the most appropriate approach currently for treatment of HD. Further developments concerning chemical modifications, dosing optimalizations, and delivery methods offer opportunities for increase in therapeutic benefit. Due to the lack of current treatment opportunities for HD is a fast available treatment option preferable. The advanced developments in non-selective approaches and seemingly positive risk-benefit profile suggest that this approach is currently preferable. The inefficacy in reducing mHTT during the allele selective ASO clinical trial of Wave life Science suggest that currently available techniques for selectivity come at the cost of reduced efficacy for mHTT lowering in humans. Nonetheless do allele selective strategies remain of interest. Long-term effects of wild-type HTT lowering remain unknown, allele selective approaches are less likely to cause long term side effects. Further development and improvements for allele selective approaches could enable a treatment circumventing long term risks.

The various approaches which enable RNA targeting of mHTT all show promise for translation into clinical beneficial therapies. Each approach has its own strengths and weaknesses. Small molecule approaches offer an ideal option for the treatment of HD. Orally availability and widespread action of small molecules make them an attractive approach. However small molecules bring greater risks of possible off target effects compared to ASOs and RNA interference. The recent developments for small molecule splicing modulators lowering mHTT level show great promise for an effective treatment. However, much more research is required before these can be applicable in a clinical setting.

ASOs are able to target the mHTT RNA and pre-RNA with a high specificity[37]. Their ability to target the RNA at a more upstream site of action compared to interference RNA presents several

advantages for their usage. Targeting pre-RNA reduces the amount of toxic variants created during splicing events of mHTT pre-RNA, diminishing more alternative pathological mechanisms of HD[21]. Furthermore, enables targeting of pre-RNA a broader range of potential targets. Both introns and exons can be targeted with ASOs[37]. A broader target range allows for more opportunities in finding an efficient and selective target. Especially allele-selective ASO approaches using SNPs as target sequence benefit from this broader target range. Only a small portion of validated SNPs of HTT pre-RNA are coding, therefore unavailable for RNA interference which targets processed RNA[51]. ASOs do not cross the blood brain barrier and are therefore delivered directly into the central nervous system intrathecal. Repetitive administration is required to maintain therapeutic effects, since ASOs are eventually depleted. This method of delivery enable the possibility to discontinue the treatment if such need arises, increasing the safety profile of ASOs. The repeated intrathecal administration can be quite invasive for the patient and increase risks of infection associated with injection into the central nervous system[32]. Viral delivery of interference RNA are more advantageous in this aspect. Even though RNA interference requires a highly invasive injection directly into the deeper brain structures, there is limited need for repetitive administration. Expression of the interference RNA is maintained for a long period of time[84]. This elevates the burden of repetitive administration for HD patients, however increases risks concerning the inability to stop the treatment.

One of the main difference between ASO and RNA interference therapy is their main side of action. ASOs distribute mainly towards subcortical structures after intrathecal injection, at these locations their mHTT lowering is the most extensive[38,54]. RNA interference are injected in the deeper brain structures and distribute only locally[82,84]. Their mHTT lowering effect is most prominent in the cortical brain structures. It is unclear which target area is most effective for therapeutic benefit. The pathology of HD disease effects the whole brain. However neuropathology occurs most prominent and in the earliest stage in the deeper brain structures such as the striatum[36]. This suggest that RNA interference in this target area has the most therapeutic benefit. However a transgenic mouse model, which expressed mHTT in either cortex or striatum, showed that reduction of mHTT in the cortex was more beneficial[89]. Indicating a higher therapeutic benefit when ASOs target cortical structures. The ongoing clinical trial of AMT-130 will give important insight in the effectiveness of targeting subcortical structures. The transgenic mouse model, which expressed mHTT in either cortex or striatum, showed the greatest benefit if both areas are targeted for mHTT reduction[89]. Further indicating that HD pathology spreads throughout the whole brain and is ideally targeted throughout the whole brain. Possible combinations of ASOs and RNA interference would provide an overlapping distribution, however could increase additional adverse effects due to addition of side effects of two types of therapies.

 Future developments of both ASO and RNA interference approaches should be targeted at less invasive delivery and more widespread distribution. Developments for ASOs, through novel chemical modifications and nanocarrier formulations, could enable a more widespread effect of this class of drug, thereby increasing their effectiveness[90]. Vector delivery of RNA interference have been developing rapidly.Novel viral vector designs can enable intravenous injection while maintain widespread distribution throughout the brain[91,92]. Therapies targeting RNA for mHTT lowering show great potential to be therapeutically beneficial for HD patients. Further research is needed for their optimization, but their advanced development and seemingly positive risk-benefit profile gives potential for a first treatment option for HD patients in the near future.

References

1. Shannon KM. Recent Advances in the Treatment of Huntington's Disease: Targeting DNA and RNA. *CNS Drugs*. 2020;34(3):219-228. doi:10.1007/S40263-019-00695-3/FIGURES/1

2. Rawlins MD, Wexler NS, Wexler AR, et al. The Prevalence of Huntington's Disease. *Neuroepidemiology*. 2016;46(2):144-153. doi:10.1159/000443738

3. Riccò M, Vezzosi L, Balzarini F, Gualerzi G, Ranzieri S. Prevalence of Huntington Disease in Italy: a systematic review and meta-analysis. *Acta Bio Medica Atenei Parm*. 2020;91(Suppl 3):119. doi:10.23750/ABM.V91I3-S.9441

4. MacDonald ME, Ambrose CM, Duyao MP, et al. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*. 1993;72(6):971-983. doi:10.1016/0092-8674(93)90585-E

5. Caron NS, Dorsey ER, Hayden MR. Therapeutic approaches to Huntington disease: from the bench to the clinic. *Nat Rev Drug Discov 2018 1710*. 2018;17(10):729-750. doi:10.1038/NRD.2018.133

6. Snell' RG, Macmillan1 JC, Cheadle' JP, et al. Relationship between trinucleotide repeat expansion and phenotypic variation in Huntington's disease. Published online 1993. Accessed January 22, 2022. http://www.nature.com/naturegenetics

7. Podvin S, Reardon HT, Yin K, Mosier C, Hook V. Multiple clinical features of Huntington's disease correlate with mutant HTT gene CAG repeat lengths and neurodegeneration. *J Neurol*. 2019;266(3):551-564. doi:10.1007/S00415-018-8940-6/FIGURES/4

8. Nasir J, Floresco SB, O JR, et al. Targeted Disruption of the Huntington's Disease Gene Results in Embryonic Lethality and Behavioral and Morphological Changes in Heterozygotes. *Cell*. 1995;81:811-823.

9. Cattaneo E, Zuccato C, Tartari M. Normal huntingtin function: an alternative approach to Huntington's disease. *Nat Rev Neurosci 2005 612*. 2005;6(12):919-930. doi:10.1038/NRN1806

10. Zuccato C, Ciammola A, Rigamonti D, et al. Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science (80-)*. 2001;293(5529):493-498. doi:10.1126/SCIENCE.1059581/SUPPL_FILE/1059581S3_THUMB.GIF

11. Zuccato C, Tartari M, Crotti A, et al. Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat Genet 2003 351*. 2003;35(1):76-83. doi:10.1038/NG1219

12. Gunawardena S, Her LS, Brusch RG, et al. Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in Drosophila. *Neuron*. 2003;40(1):25-40. doi:10.1016/S0896-6273(03)00594-4/ATTACHMENT/E5314E08-E45C-4F0B-BEA0-558CCABD0B60/MMC5.MOV

13. Block-Galarza J, Chase KO, Sapp E, et al. Fast transport and retrograde movement of huntingtin and HAP 1 in axons. *Neuroreport*. 1997;8(9-10):2247-2251. doi:10.1097/00001756-199707070-00031

14. Smith R, Brundin P, Li JY. Synaptic dysfunction in Huntington's disease: a new perspective. *Cell Mol Life Sci C 2005 6217*. 2005;62(17):1901-1912. doi:10.1007/S00018-005-5084-5

15. Ferrante RJ, Gutekunst CA, Persichetti F, et al. Heterogeneous Topographic and Cellular Distribution of Huntingtin Expression in the Normal Human Neostriatum. *J Neurosci*. 1997;17(9):3052. doi:10.1523/JNEUROSCI.17-09-03052.1997

16. Heinz A, Nabariya DK, Krauss S. Huntingtin and Its Role in Mechanisms of RNA-Mediated Toxicity. *Toxins (Basel)*. 2021;13(7). doi:10.3390/TOXINS13070487

17. Song W, Chen J, Petrilli A, et al. MUTANT HUNTINGTIN BINDS THE MITOCHONDRIAL FISSION GTPASE DRP1 AND INCREASES ITS ENZYMATIC ACTIVTY. *Nat Med*. 2011;17(3):377. doi:10.1038/NM.2313

18. DiFiglia M, Sapp E, Chase KO, et al. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science (80-)*. 1997;277(5334):1990-1993. doi:10.1126/SCIENCE.277.5334.1990/ASSET/7433F340-81EB-4536-932B-F1DD8E97A9B0/ASSETS/GRAPHIC/SE3975752005.JPEG

19. Arrasate M, Finkbeiner S. Protein aggregates in Huntington's disease. *Exp Neurol*. 2012;238(1):1. doi:10.1016/J.EXPNEUROL.2011.12.013

20. Neueder A, Landles C, Ghosh R, et al. The pathogenic exon 1 HTT protein is produced by incomplete splicing in Huntington's disease patients. *Sci Rep*. 2017;7(1). doi:10.1038/S41598-017-01510-Z

21. Bañez-Coronel M, Porta S, Kagerbauer B, et al. A Pathogenic Mechanism in Huntington's Disease Involves Small CAG-Repeated RNAs with Neurotoxic Activity. *PLoS Genet*. 2012;8(2):1002481. doi:10.1371/JOURNAL.PGEN.1002481

22. Zu T, Gibbens B, Doty NS, et al. Non-ATG-initiated translation directed by microsatellite expansions. *Proc Natl Acad Sci U S A*. 2011;108(1):260-265. doi:10.1073/PNAS.1013343108/-/DCSUPPLEMENTAL

23. Bañez-Coronel M, Ayhan F, Tarabochia AD, et al. RAN Translation in Huntington Disease. *Neuron*. 2015;88(4):667. doi:10.1016/J.NEURON.2015.10.038

24. Rudich P, Watkins S, Lamitina T. PolyQ-independent toxicity associated with novel translational products from CAG repeat expansions. *PLoS One*. 2020;15(4). doi:10.1371/JOURNAL.PONE.0227464

25. Yang S, Yang H, Huang L, et al. Lack of RAN-mediated toxicity in Huntington's disease knock-in mice. *Proc Natl Acad Sci U S A*. 2020;117(8):4411-4417. doi:10.1073/PNAS.1919197117/-/DCSUPPLEMENTAL

26. Jiang R, Diaz-Castro B, Looger LL, Khakh BS. Dysfunctional Calcium and Glutamate Signaling in Striatal Astrocytes from Huntington's Disease Model Mice. *J Neurosci*. 2016;36(12):3453. doi:10.1523/JNEUROSCI.3693-15.2016

27. Tong X, Ao Y, Faas GC, et al. Astrocyte Kir4.1 ion channel deficits contribute to neuronal dysfunction in Huntington's disease model mice. *Nat Neurosci*. 2014;17(5):694. doi:10.1038/NN.3691

28. Gauthier LR, Charrin BC, Borrell-Pagès M, et al. Huntingtin Controls Neurotrophic Support and Survival of Neurons by Enhancing BDNF Vesicular Transport along Microtubules. *Cell*. 2004;118(1):127-138. doi:10.1016/J.CELL.2004.06.018

29. Crotti A, Benner C, Kerman BE, et al. Mutant Huntingtin promotes autonomous microglia activation via myeloid lineage-determining factors. *Nat Neurosci*. 2014;17(4):513. doi:10.1038/NN.3668

30. Yamamoto A, Lucas JJ, Hen R. Reversal of Neuropathology and Motor Dysfunction in a Conditional Model of Huntington's Disease. *Cell*. 2000;101(1):57-66. doi:10.1016/S0092-8674(00)80623-6

31.   Wang N, Gray M, Lu XH, et al. Neuronal Targets of Mutant Huntingtin Genetic Reduction to Ameliorate Huntington's Disease Pathogenesis in Mice. *Nat Med*. 2014;20(5):536. doi:10.1038/NM.3514

32.   Tabrizi SJ, Ghosh R, Leavitt BR. Huntingtin Lowering Strategies for Disease Modification in Huntington's Disease. *Neuron*. 2019;101(5):801-819. doi:10.1016/J.NEURON.2019.01.039

33.   Ambrose CM, Duyao MP, Barnes G, et al. Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. *Somat Cell Mol Genet*. 1994;20(1):27-38. doi:10.1007/BF02257483

34.   Rodan LH, Cohen J, Fatemi A, et al. A novel neurodevelopmental disorder associated with compound heterozygous variants in the huntingtin gene. *Eur J Hum Genet*. 2016;24(12):1826. doi:10.1038/EJHG.2016.74

35.   Duyao MP, Auerbach AB, Ryan A, et al. Inactivation of the Mouse Huntington's Disease Gene Homolog Hdh. *Science (80-)*. 1995;269(5222):407-410. doi:10.1126/SCIENCE.7618107

36.   De Souza RAG, Leavitt BR. Neurobiology of Huntington's Disease. *Curr Top Behav Neurosci*. 2014;22:81-100. doi:10.1007/7854_2014_353

37.   Bennett CF, Swayze EE. RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform INTRODUCTION: AN RNA WORLD. *Annu Rev Pharmacol Toxicol is Annu Rev Pharmacol Toxicol*. 2010;50:259-293. doi:10.1146/annurev.pharmtox.010909.105654

38.   Kordasiewicz HB, Stanek LM, Wancewicz E V., et al. Sustained therapeutic reversal of Huntington's disease by transient repression of huntingtin synthesis. *Neuron*. 2012;74(6):1031. doi:10.1016/J.NEURON.2012.05.009

39.   Southwell AL, Skotte NH, Bennett CF, Hayden MR. Antisense oligonucleotide therapeutics for inherited neurodegenerative diseases. *Trends Mol Med*. 2012;18(11):634-643. doi:10.1016/J.MOLMED.2012.09.001

40.   Jarosińska OD, Rüdiger SGD. Molecular Strategies to Target Protein Aggregation in Huntington's Disease. *Front Mol Biosci*. 2021;8. doi:10.3389/FMOLB.2021.769184

41.   Aslesh T, Yokota T. Development of Antisense Oligonucleotide Gapmers for the Treatment of Huntington's Disease. *Methods Mol Biol*. 2020;2176:57-67. doi:10.1007/978-1-0716-0771-8_4

42.   Liang XH, Sun H, Nichols JG, Crooke ST. RNase H1-Dependent Antisense Oligonucleotides Are Robustly Active in Directing RNA Cleavage in Both the Cytoplasm and the Nucleus. *Mol Ther*. 2017;25(9):2075-2092. doi:10.1016/J.YMTHE.2017.06.002

43.   Gray M, Shirasaki DI, Cepeda C, et al. Full-Length Human Mutant Huntingtin with a Stable Polyglutamine Repeat Can Elicit Progressive and Selective Neuropathogenesis in BACHD Mice. *J Neurosci*. 2008;28(24):6182. doi:10.1523/JNEUROSCI.0857-08.2008

44.   Slow EJ, van Raamsdonk J, Rogers D, et al. Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. *Hum Mol Genet*. 2003;12(13):1555-1567. doi:10.1093/HMG/DDG169

45.   Dragatsis I, Levine MS, Zeitlin S. Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. *Nat Genet 2000 263*. 2000;26(3):300-306. doi:10.1038/81593

46.   Hu J, Matsui M, Gagnon KT, et al. Allele-specific silencing of mutant huntingtin and ataxin-3

genes by targeting expanded CAG repeats in mRNAs. *Nat Biotechnol 2009 275*. 2009;27(5):478-484. doi:10.1038/NBT.1539

47. Gagnon KT, Pendergraff HM, Deleavey GF, et al. Allele-selective inhibition of mutant huntingtin expression with antisense oligonucleotides targeting the expanded CAG repeat. *Biochemistry*. 2010;49(47):10166-10178. doi:10.1021/BI101208K/SUPPL_FILE/BI101208K_SI_001.PDF

48. Monia BP, Johnston JF, Ecker DJ, Zounes MA, Lima WF, Freier SM. Selective inhibition of mutant Ha-ras mRNA expression by antisense oligonucleotides. *J Biol Chem*. 1992;267(28):19954-19962. doi:10.1016/S0021-9258(19)88650-7

49. Warby SC, Montpetit A, Hayden AR, et al. CAG Expansion in the Huntington Disease Gene Is Associated with a Specific and Targetable Predisposing Haplogroup. *Am J Hum Genet*. 2009;84(3):351. doi:10.1016/J.AJHG.2009.02.003

50. Pfister EL, Kennington L, Straubhaar J, et al. Five siRNAs Targeting Three SNPs May Provide Therapy for Three-Quarters of Huntington's Disease Patients. *Curr Biol*. 2009;19(9):774-778. doi:10.1016/J.CUB.2009.03.030

51. Carroll JB, Warby SC, Southwell AL, et al. Potent and Selective Antisense Oligonucleotides Targeting Single-Nucleotide Polymorphisms in the Huntington Disease Gene / Allele-Specific Silencing of Mutant Huntingtin. *Mol Ther*. 2011;19(12):2178. doi:10.1038/MT.2011.201

52. Hodgson JG, Smith DJ, McCutcheon K, et al. Human huntingtin derived from YAC transgenes compensates for loss of murine huntingtin by rescue of the embryonic lethal phenotype. *Hum Mol Genet*. 1996;5(12):1875-1885. doi:10.1093/HMG/5.12.1875

53. Southwell AL, Warby SC, Carroll JB, et al. A fully humanized transgenic mouse model of Huntington disease. *Hum Mol Genet*. 2013;22(1):18-34. doi:10.1093/HMG/DDS397

54. Southwell AL, Skotte NH, Kordasiewicz HB, et al. In Vivo Evaluation of Candidate Allele-specific Mutant Huntingtin Gene Silencing Antisense Oligonucleotides. *Mol Ther*. 2014;22(12):2093. doi:10.1038/MT.2014.153

55. Tabrizi SJ, Leavitt BR, Landwehrmeyer GB, et al. Targeting Huntingtin Expression in Patients with Huntington's Disease. *N Engl J Med*. 2019;380(24):2307-2316. doi:10.1056/NEJMOA1900907/SUPPL_FILE/NEJMOA1900907_DATA-SHARING.PDF

56. McColgan P. Roche Community Presentation of New Tominersen Data. Huntington' s disease association of America. Accessed February 10, 2022. https://www.youtube.com/watch?v=41vu1M8xpkQ

57. Wave Life Sciences Provides Update on Phase 1b/2a PRECISION-HD Trials - Wave Life Sciences. Accessed February 10, 2022. https://ir.wavelifesciences.com/news-releases/news-release-details/wave-life-sciences-provides-update-phase-1b2a-precision-hd

58. Boudreau RL, Martins I, Davidson BL. Artificial MicroRNAs as siRNA Shuttles: Improved Safety as Compared to shRNAs In vitro and In vivo. *Mol Ther J Am Soc Gene Ther*. 2008;17(1):169. doi:10.1038/MT.2008.231

59. De Fougerolles AR. Delivery Vehicles for Small Interfering RNA In Vivo. *https://home-liebertpub-com.proxy.library.uu.nl/hum*. 2008;19(2):125-132. doi:10.1089/HUM.2008.928

60. Miniarikova J, Evers MM, Konstantinova P. Translation of MicroRNA-Based Huntingtin-Lowering Therapies from Preclinical Studies to the Clinic. *Mol Ther*. 2018;26(4):947-962. doi:10.1016/J.YMTHE.2018.02.002

61.   Naso MF, Tomkowicz B, Perry WL, Strohl WR. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *Biodrugs*. 2017;31(4):317. doi:10.1007/S40259-017-0234-5

62.   Kantor B, McCown T, Leone P, Gray SJ. Clinical Applications Involving CNS Gene Transfer. *Adv Genet*. 2014;87:71. doi:10.1016/B978-0-12-800149-3.00002-0

63.   Wang YL, Liu W, Wada E, Murata M, Wada K, Kanazawa I. Clinico-pathological rescue of a model mouse of Huntington's disease by siRNA. *Neurosci Res*. 2005;53(3):241-249. doi:10.1016/J.NEURES.2005.06.021

64.   DiFiglia M, Sena-Esteves M, Chase K, et al. Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. *Proc Natl Acad Sci U S A*. 2007;104(43):17204. doi:10.1073/PNAS.0708285104

65.   Stiles DK, Zhang Z, Ge P, et al. Widespread suppression of huntingtin with convection-enhanced delivery of siRNA. *Exp Neurol*. 2012;233(1):463-471. doi:10.1016/J.EXPNEUROL.2011.11.020

66.   Grondin R, Ge P, Chen Q, et al. Onset Time and Durability of Huntingtin Suppression in Rhesus Putamen After Direct Infusion of Antihuntingtin siRNA. *Mol Ther Nucleic Acids*. 2015;4(6):e245. doi:10.1038/MTNA.2015.20

67.   Boudreau RL, Monteys AM, Davidson BL. Minimizing variables among hairpin-based RNAi vectors reveals the potency of shRNAs. *RNA*. 2008;14(9):1834. doi:10.1261/RNA.1062908

68.   Chen ZJ, Kren BT, Wong PYP, Low WC, Steer CJ. Sleeping Beauty-mediated down-regulation of huntingtin expression by RNA interference. *Biochem Biophys Res Commun*. 2005;329(2):646-652. doi:10.1016/J.BBRC.2005.02.024

69.   Harper SQ, Staber PD, He X, et al. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. Published online 2005. Accessed February 15, 2022. www.pnas.orgcgidoi10.1073pnas.0501507102

70.   Kaye J, Reisine T, Finkbeiner S. Huntington's disease mouse models: unraveling the pathology caused by CAG repeat expansion. *Fac Rev*. 2021;10(77). doi:10.12703/R/10-77

71.   Rodriguez-Lebron E, Denovan-Wright EM, Nash K, Lewin AS, Mandel RJ. Intrastriatal rAAV-Mediated Delivery of Anti-huntingtin shRNAs Induces Partial Reversal of Disease Progression in R6/1 Huntington's Disease Transgenic Mice. *Mol Ther*. 2005;12(4):618. doi:10.1016/J.YMTHE.2005.05.006

72.   Machida Y, Okada T, Kurosawa M, Oyama F, Ozawa K, Nukina N. rAAV-mediated shRNA ameliorated neuropathology in Huntington disease model mouse. *Biochem Biophys Res Commun*. 2006;343(1):190-197. doi:10.1016/J.BBRC.2006.02.141

73.   Drouet V, Perrin V, Hassig R, et al. Sustained effects of nonallele-specific Huntingtin silencing. *Ann Neurol*. 2009;65(3):276-285. doi:10.1002/ANA.21569

74.   Mcbride JL, Boudreau RL, Harper SQ, et al. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: Implications for the therapeutic development of RNAi. Published online 2008. Accessed February 15, 2022. www.pnas.org/cgi/content/full/

75.   Boudreau RL, McBride JL, Martins I, et al. Nonallele-specific Silencing of Mutant and Wild-type Huntingtin Demonstrates Therapeutic Efficacy in Huntington's Disease Mice. *Mol Ther*. 2009;17(6):1053-1063. doi:10.1038/MT.2009.17

76.   Stanek LM, Sardi SP, Mastis B, et al. Silencing mutant huntingtin by adeno-associated virus-mediated RNA interference ameliorates disease manifestations in the YAC128 mouse model

of Huntington's Disease. *Hum Gene Ther*. 2014;25(5):461-474. doi:10.1089/HUM.2013.200/ASSET/IMAGES/LARGE/FIGURE6.JPEG

77. McBride JL, Pitzer MR, Boudreau RL, et al. Preclinical Safety of RNAi-Mediated HTT Suppression in the Rhesus Macaque as a Potential Therapy for Huntington's Disease. *Mol Ther*. 2011;19(12):2152-2162. doi:10.1038/MT.2011.219

78. Grondin R, Kaytor MD, Ai Y, et al. Six-month partial suppression of Huntingtin is well tolerated in the adult rhesus striatum. *Brain*. 2012;135(4):1197-1209. doi:10.1093/BRAIN/AWR333

79. Miniarikova J, Zanella I, Huseinovic A, et al. Design, Characterization, and Lead Selection of Therapeutic miRNAs Targeting Huntingtin for Development of Gene Therapy for Huntington's Disease. *Mol Ther - Nucleic Acids*. 2016;5:e297. doi:10.1038/MTNA.2016.7

80. Miniarikova J, Zimmer V, Martier R, et al. AAV5-miHTT gene therapy demonstrates suppression of mutant huntingtin aggregation and neuronal dysfunction in a rat model of Huntington's disease. *Gene Ther 2017 2410*. 2017;24(10):630-639. doi:10.1038/GT.2017.71

81. Drouet V, Ruiz M, Zala D, et al. Allele-Specific Silencing of Mutant Huntingtin in Rodent Brain and Human Stem Cells. *PLoS One*. 2014;9(6):e99341. doi:10.1371/JOURNAL.PONE.0099341

82. Pfister EL, Dinardo N, Mondo E, et al. Artificial miRNAs Reduce Human Mutant Huntingtin Throughout the Striatum in a Transgenic Sheep Model of Huntington's Disease. *Hum Gene Ther*. 2018;29(6):663-673. doi:10.1089/HUM.2017.199/SUPPL_FILE/SUPP_TABLE4.PDF

83. Evers MM, Miniarikova J, Juhas S, et al. AAV5-miHTT Gene Therapy Demonstrates Broad Distribution and Strong Human Mutant Huntingtin Lowering in a Huntington's Disease Minipig Model. *Mol Ther*. 2018;26(9):2163. doi:10.1016/J.YMTHE.2018.06.021

84. Spronck EA, Brouwers CC, Vallès A, et al. AAV5-miHTT Gene Therapy Demonstrates Sustained Huntingtin Lowering and Functional Improvement in Huntington Disease Mouse Models. *Mol Ther Methods Clin Dev*. 2019;13:334. doi:10.1016/J.OMTM.2019.03.002

85. Phase I/II Clinical Trial of AMT-130 - Patients - uniQure. Accessed February 16, 2022. http://www.uniqure.com/patients/phase-1-2-clinical-trial-of-amt-130.php

86. Voyager Therapeutics I. Voyager Therapeutics Transforms Pipeline and Increases Investment in Next-Generation TRACER™ AAV Capsid and Vectorized Antibody Platform Technologies. Published online 2021. Accessed February 16, 2022. https://ir.voyagertherapeutics.com/news-releases/news-release-details/voyager-therapeutics-transforms-pipeline-and-increases/

87. Bhattacharyya A, Trotta CR, Narasimhan J, et al. Small molecule splicing modifiers with systemic HTT-lowering activity. *Nat Commun 2021 121*. 2021;12(1):1-12. doi:10.1038/S41467-021-27157-Z

88. Southwell AL, Kordasiewicz HB, Langbehn D, et al. Huntingtin suppression restores cognitive function in a mouse model of Huntington's disease. *Sci Transl Med*. 2018;10(461):3959. doi:10.1126/SCITRANSLMED.AAR3959/SUPPL_FILE/AAR3959_TABLE_S6.XLSX

89. Wang N, Gray M, Lu XH, et al. Neuronal Targets of Mutant Huntingtin Genetic Reduction to Ameliorate Huntington's Disease Pathogenesis in Mice. *Nat Med*. 2014;20(5):536. doi:10.1038/NM.3514

90. Su Min H, yun Jin Kim H, Naito M, et al. Systemic Brain Delivery of Antisense Oligonucleotides across the Blood–Brain Barrier with a Glucose-Coated Polymeric Nanocarrier. *Angew Chemie*. 2020;132(21):8250-8257. doi:10.1002/ANGE.201914751

91.   Dufour BD, Smith CA, Clark RL, Walker TR, Mcbride JL. Intrajugular Vein Delivery of AAV9-RNAi Prevents Neuropathological Changes and Weight Loss in Huntington's Disease Mice. *Mol Ther*. 2014;22(4):797. doi:10.1038/MT.2013.289

92.   Dash D, Mestre TA. Therapeutic Update on Huntington's Disease: Symptomatic Treatments and Emerging Disease-Modifying Therapies. *Neurotherapeutics*. 2020;17(4):1645. doi:10.1007/S13311-020-00891-W