AAV5-miHTT gene therapy demonstrates sustained huntingtin lowering and functional improvement in Huntington disease mouse models

Authors: Elisabeth A. Spronck¹, Cynthia C. Brouwers¹, Astrid Vallès¹, Martin de Haan¹, Harald Petry¹, Sander J. van Deventer¹², Pavlina Konstantinova¹, Melvin M. Evers¹∗

Affiliations:

¹Department of Research & Development, uniQure biopharma B.V., Amsterdam, the Netherlands

²Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands

Keywords: Huntington disease, gene silencing, HD mouse model, gene therapy, therapeutic microRNAs, adeno-associated viral vector serotype 5

*Correspondence should be addressed to Dr. Melvin Evers

Email: m.evers@unique.com
Abstract

Huntington disease (HD) is a fatal neurodegenerative disorder caused by an autosomal dominant CAG repeat expansion in the huntingtin (HTT) gene. The translated expanded polyglutamine repeat in the huntingtin protein is known to cause toxic gain-of-function. We previously showed that strong huntingtin lowering prevented neuronal dysfunction in HD rodents and minipigs after single intra-cranial injection of an adeno-associated viral vector serotype 5 expressing a microRNA targeting human HTT (AAV5-miHTT). To evaluate long-term efficacy, knock-in Q175 HD mice were injected into the striatum with AAV5-miHTT and sacrificed 12 months post-injection. AAV5-miHTT caused a dose-dependent and sustained huntingtin protein reduction with subsequent suppression of mutant huntingtin aggregate formation in the striatum and cortex. Functional proof-of-concept was shown in transgenic R6/2 HD mice. Eight weeks after AAV5-miHTT treatment, a significant improvement in motor coordination on the rotarod was observed. Survival analysis showed that a single AAV5-miHTT treatment resulted in a significant four week increase in median survival compared with vehicle-treated R6/2 HD mice. The combination of long-term huntingtin lowering, reduction of aggregation, prevention of neuronal dysfunction, alleviation of HD-like symptoms and beneficial survival observed in HD rodents treated with AAV5-miHTT support the continued development of huntingtin-lowering gene therapies for HD.
Introduction

Huntington disease (HD) is a neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the first exon of the huntingtin (HTT) gene. This results in an expanded polyglutamine repeat in the huntingtin protein, causing toxic gain-of-function and affecting numerous cellular processes and ultimately leading to neuronal death.\(^1\) Disease onset usually occurs around 35-45 years of age and symptoms are choreiform movement, impaired coordination and psychiatric disturbances.\(^2\) HD is a fatal disease, usually within 15 years after disease onset.\(^3\) Brain areas that are affected most by the disease are the striatum and the motor cortex.\(^4\) Clearance of the mutant huntingtin is currently accepted as being key for HD treatment and is the target of several different potential disease modifying therapies.\(^5\)

Since the discovery of the HTT gene, several groups have investigated huntingtin lowering strategies such as antisense oligonucleotides (ASOs), RNA interference (RNAi), ribozymes, DNA enzymes, and genome-editing approaches.\(^6\),\(^7\) One of therapeutic approaches for huntingtin lowering is to act through binding of molecules to HTT mRNA to block the translation into the toxic huntingtin protein. ASOs have been shown to lower the amount of huntingtin protein in mouse models of HD which lead to delayed disease progression and even reversal of disease phenotype.\(^4\),\(^8\),\(^9\) Currently, a phase I trial using repetitive intrathecal administration of ASOs in HD patients is ongoing.\(^10\) Artificial small interfering (si)RNAs, short-hairpin (sh)RNAs, or micro (mi)RNAs bind to the HTT mRNA and reduce its translation by the endogenous RNAi machinery.\(^11\) Adeno-associated viral (AAV) vectors are the most common vehicles of choice to deliver the gene cassette containing RNAi and a large number of AAV capsid serotypes provide cell- and tissue- specific tropism.\(^12\) For the central nervous system (CNS), studies in rodents and
non-human primates have shown AAV serotype 5 (AAV5) to effectively transduce the brain, making it an attractive candidate for RNAi-based gene transfer.\textsuperscript{13-16} Our approach involves expression of a gene cassette encoding an engineered miRNA targeting human HTT, delivered via AAV5 (AAV5-miHTT) directly into the brain area affected most in HD, the striatum. This would allow for continuous expression of therapeutic miRNAs after a single administration of the AAV vector, potentially resulting in long-term HTT lowering.

We previously showed strong reduction of huntingtin in the brain of humanized HD mice,\textsuperscript{17} prevention of neuronal dysfunction in lentiviral HD rats after single intracranial injection of AAV5-miHTT \textsuperscript{18} and successful translation to the large HD minipig brain.\textsuperscript{19} Here, we have investigated long-term huntingtin protein lowering, tolerability of AAV5-miHTT treatment and functional improvement in the Q175 Knock In (KI) (heterozygous) HD mice \textsuperscript{20} and the R6/2 HD mice.\textsuperscript{21} The models were chosen to investigate both the slow disease progression in the Q175 mice as well as the rapidly developing phenotype seen in the R6/2 model. Both models were one-time treated with AAV-miHTT directly into the striatum. A dose-dependent, sustained huntingtin protein reduction with subsequent suppression of mutant huntingtin aggregate formation in the striatum and cortex was found in the Q175 mice. The R6/2 mice showed functional improvement 8 weeks after AAV5-miHTT treatment. One-time AAV5-miHTT administration resulted in a median survival improvement of over 4 weeks, when compared to untreated R6/2 HD mice. The extensive human mutant huntingtin lowering, functional improvement, survival benefit and tolerability of AAV5-miHTT, supports further development of our huntingtin-lowering gene therapy and initiation of clinical trials in HD patients in the near future.
Results

One-time intra-striatal AAV5-miHTT administration results in long-term expression of miHTT and huntingtin lowering in Q175 KI mice

We have previously demonstrated strong suppression of huntingtin and improved neuropathology in HD rodents using miHTT, a miRNA targeting human HTT exon 1 expressed from a one-time delivery of AAV5 gene therapy.\textsuperscript{17,18} The current studies were conducted to investigate the long-term expression and efficacy of miHTT in a mouse model of HD with a behavioral phenotype. In the heterozygous Q175 KI mice, the murine HTT exon 1 and part of intron 1 have been replaced by the human counterparts. The human exon 1 contains a large CAG repeat, which allows to study the mechanism-of-action of AAV5-miHTT. To determine the long-term expression of the transgene and subsequent huntingtin lowering, adult Q175 KI heterozygous mice were injected bilaterally in the striatum with AAV5-miHTT at 5x increasing doses (n=8, males only; low, mid and high dose). Additional wild-type littermates and Q175 KI mice were injected with vehicle and served as control groups (Figure 1A). To evaluate any phenotypical changes, behavioral analyses were performed at several time points following administration of AAV5-miHTT. The behavioral analysis consisted of Rotarod, open field and fine motor analysis (MotoRater), evaluated every 3 months (Figure 1B). Twelve months after the administration of AAV5-miHTT the right hemisphere of the brains from all animals were taken to determine the level of vector DNA, transgene RNA and huntingtin protein (Figure 1C). The striatum and cortex samples were used to determine the amount of huntingtin protein lowering to assess target engagement. Distribution of the vector DNA and transgene expression were determined in the cortex samples.
Vector DNA distribution and expression of the miRNA targeting HTT mRNA in the brain of Q175 KI mice

The amount of vector DNA in the cortex of Q175 KI mice was determined by quantitative PCR using primers designed specifically for the AAV5-miHTT expression cassette. Insufficient striatum tissue was available for this analysis due to the limited size of the mouse striatum. Even though the cortex was not the target region of the intra-striatal injection, dose dependent levels of vector DNA were observed in the cortex of AAV5-miHTT treated animals (Figure 2A). The low dose showed $1.3 \times 10^4$ vector genome copies per µg genomic DNA (gc/µg gDNA), while the high dose animals had values of up to $10^6$ gc/µg gDNA. As expected, vehicle-treated animals all gave values below background levels. Guide miHTT transgene (relative to vehicle treated animals) was also dose-dependently expressed in the cortex of AAV5-miHTT treated animals. Relative to vehicle-treated animals, expression of the transgene was on average of 200, 1500 and 4000-fold higher in the low, mid and high dose animals, respectively. Vector DNA levels significantly correlate with the level of miHTT expression in the cortex (supplementary figure 1). Vector DNA as well as dose-dependent miHTT expression in the cortex were sustained 12 months after a striatal injection of AAV5-miHTT in Q175 mice. Since the miHTT transgene is expressed in the cortex, an area of the brain that was not the direct target of the AAV5-miHTT administration, this indicated that the AAV5 vector was actively distributed via axonal transport through the brain. GFP expression in mouse brains injected with AAV5-GFP following the same administration and dose range also shows dose-dependent widespread distribution of the vector transgene to the striatum and cortex (supplementary figure 2).
Dose dependent huntingtin protein and aggregate lowering in the brain of het Q175 KI mice

The striatum and cortex from the right hemisphere were used to determine the expression of soluble human mutant huntingtin protein. A significant dose-dependent average reduction of mutant huntingtin protein of up to 39% in the striatum and up to 13% in the cortex of Q175 KI, 12 months after intra-striatal injection of AAV5-miHTT was detected (Figure 3A). The left hemisphere was used for EM48 staining to determine the amount of mutant huntingtin aggregates in the striatum and cortex, the pathological hallmark of HD (Figure 3B). The EM48 staining showed a reduction of huntingtin aggregates in the striatum and cortex of Q175 KI mice treated with the high dose, when compared to vehicle treated Q175 KI mice (Figure 3C). Both the soluble huntingtin protein and the huntingtin aggregate analysis demonstrate that AAV5-miHTT leads to sustained huntingtin lowering and reduction of aggregation in a mouse model of HD.

No clear behavioral phenotype in the Q175 KI mouse model

A rotarod and open field test were performed on the Q175 KI mice and wild-type littermates before dosing (at 3 months of age) and on consecutive intervals at 3, 6- and 9- months following administration of vehicle with AAV5-miHTT. Neither the rotarod or the open field test showed a clear phenotype when comparing the vehicle treated Q175 KI mice to the wild-type littermates (One-way ANOVA, Dunnett’s post-hoc test, p>0.05) (supplementary figure 3). This has been previously reported by others when using the heterozygous mouse model.22 Fine motor skills and gait analysis was performed using the MotoRater, which records the complete movement of the mice. The principle component analysis of this gait analysis showed minimal and inconsistent differences between Q175 KI mice and wild type littermates. Thus, due to lack of gross motor
phenotype development and absence of clear behavioral deficits in this Q175 HET KI mouse model, an improvement by AAV5-miHTT treatment on these functional parameters could not be assessed in this study.

**Long term tolerability of AAV5-miHTT treatment in het Q175 KI mice**

AAV5-miHTT intra-striatal administration led to widespread distribution, long-term expression of the therapeutic miHTT, dose-dependent knockdown of huntingtin protein and a reduction in the number of huntingtin aggregates in both the striatum and cortex. Unfortunately, no phenotype was observed so the effect of AAV5-miHTT on an HD-like phenotype could not be evaluated. As the mice were followed for 1 year, we also investigated the tolerability of long-term AAV5-miHTT treatment. At necropsy blood samples were used for clinical chemistry and AAV5-miHTT treatment did not result in any changes in the parameters that were measured (supplementary figure 4). The left hemisphere of the brain and all major organs were collected and subjected to macroscopic and microscopic examination by a certified pathologist. This revealed no treatment related findings in any of the organs, including the brain after H&E staining (data not shown). Although the heterozygous Q175 KI mice did not develop a clear HD-like phenotype, AAV5-miHTT treatment did not trigger any behavioral deficits. In conclusion, investigations looking into tolerability of long-term high vector DNA and miHTT expression show no tolerability issues, up to 1 year after the initial treatment.

**Intra-striatal AAV5-miHTT administration results in functional and survival improvement in R6/2 mice**

As the Q175 KI HD mice did not develop a clear HD-like phenotype, the effect of AAV5-miHTT treatment on the functional improvement of HD-like symptoms, was conducted in a
follow-up study in R6/2 HD mice. The R6/2 mouse was selected for the rapid phenotype and because this model expresses the expanded exon 1 of the human \textit{HTT} gene including the target site of the miHTT.\textsuperscript{15} This mouse model is known for showing symptoms as early as 6-8 weeks of age and with a median survival of approximately 16 weeks.\textsuperscript{23} The R6/2 mice were injected at 4 weeks of age with AAV5-miHTT using the same procedure and doses as the Q175 KI mouse study and followed for up to 5 months (Figure 4A). Behavior testing in the form of rotarod and evaluation of the clasping phenotype was performed at several time points during the in-life phase (Figure 4B).

The R6/2 mice are known to have impaired body weight gain during their lifespan, and in the current study R6/2 mice started losing body weight around 10 weeks of age (supplementary figure 5). Starting at 11 weeks of age R6/2 mice also had increased mortality, and consequently the number of animals per group decreased over time. Treatment with the high dose of AAV5-miHTT resulted in a significantly higher body weight in the R6/2 mice when compared to untreated R6/2 mice at 10, 11, 13, 15-19 and 21 weeks of age (one-way ANOVA, p<0.05).

R6/2 mice lose motor coordination over time and this can be monitored using the Rotarod test.\textsuperscript{21} The resulting phenotype in this model becomes severe around 8-12 weeks of age.\textsuperscript{15} In our study the rotarod test was performed at week 0 (pre-dose), 4 and 8 weeks after treatment with AAV5-miHTT. A decreased latency to fall was observed in the vehicle treated R6/2 mice already at 8 weeks of age (Figure 6A) and at 12 weeks of age this difference was even more pronounced. AAV5-miHTT treatment showed a significant improvement in motor coordination on the rotarod by an increase in the latency to fall of 22 seconds in the high dose group, which is an increase of
56% when compared to the vehicle treated R6/2 mice (One-way ANOVA, p=0.0226) shown in figure 6B.

Wild-type animals spread out their limbs when they are picked up by the tail, while R6/2 mice are known for clasping their hind and front limbs tightly against their body due to their motor deficits. Both the time until the hindlegs touched each other and the time until full clasping of the hindlegs was recorded for 5 animals per group. A dose-dependent trend of increasing the time to touch the hindlegs and to clasping was observed in the AAV5-miHTT treated animals, when compared to vehicle treated R6/2 mice at 12 weeks of age, but these results did not reach significance (Figure 6C) (One-way ANOVA).

The average lifespan of a wild type mouse is approximately 2 years, while the R6/2 mice have a median survival of 16 weeks, with increased mortality starting at 10 weeks of age. The one-time bilateral intra-striatal injection of both the low and high dose of AAV5-miHTT resulted in a significant increase in median survival compared with vehicle-treated R6/2 HD mice (Figure 7). Treating the R6/2 mice with a low dose increased the median survival by 26.5 days (p=0.035), while the high dose showed an increase of 29 days (median 149 days; p=0.0292) when compared to untreated R6/2 mice which had a median survival of 120 days.

The R6/2 mice used in the study showed a strong phenotype based on body weight, motor coordination, behavior and survival end points when compared to their wild type littermates. Our data show that treatment with AAV5-miHTT lead to significant improvements in body weight, behavior and an increased survival, thereby partially correcting the severe HD-like phenotype.
Discussion

The present study shows that targeting human huntingtin mRNA is effective both in lowering the soluble mutant huntingtin protein and reducing the amount of huntingtin aggregates in the brain, in a relevant murine model of HD. Intra-striatal administration of AAV5-miHTT resulted in therapeutic miRNA expression throughout the murine brain and huntingtin protein lowering in both the cortex and the striatum in the Q175 HD mouse model. This is in agreement with our previous publication where it was shown that AAV5-miHTT suppressed HTT mRNA and thereby prevented the formation of mutant huntingtin aggregate formation, which lead to suppression of DARPP-32 associated neuronal dysfunction in a HD rat model. The current studies confirm huntingtin protein lowering in the CNS, now up to 12 months after treatment. The huntingtin lowering is not as strong as might be expected, most likely due to differences between the assays and because the entire brain structures were used, which can dilute the effect. Nevertheless, there is still an almost 40% reduction of huntingtin protein in the striatum. In addition, we have shown functional improvement in a severe mouse model for HD after treatment with AAV5-miHTT which is seen in the rotarod test, the body weight and improved median survival of the R6/2 mouse model.

The heterozygous Q175 KI model was chosen for our study because the model contains an expanded CAG repeat within one of the native mouse huntingtin genes, which mimics the mutation found in HD patients and is therefore a reliable measure of huntingtin suppression. However, recently, it has been shown that this model does not show a robust HD-like phenotype. A newer model, the Q175FDN mice has now been recommended as a more relevant HD model. Hence, heterozygous Q175 mice could not be used for evaluation of motor
function defects, and of phenotype improvement by AAV5-miHTT treatment. However, the study does show that a one-time intra-striatal administration of AAV5-miHTT resulted in a dose-dependent lowering of the huntingtin protein 12 months following treatment. This reduction of mutant huntingtin was observed both in the cortex and the striatum, and resulted in reduction of the number of huntingtin aggregates. For this analysis a homogenized sample from each structure was used to determine the level of huntingtin protein. This does not allow for the determination of huntingtin protein levels in a specific brain area and could dilute the actual huntingtin lowering effect in some areas, particularly in the cortex. The safety of AAV5-miHTT treatment and consequent huntingtin lowering was also evaluated in this study and no adverse effects, effects on clinical chemistry or histopathological changes in any of the major organs, including the brain, were found, indicating long-term tolerability of a one-time administration of AAV5-miHTT to the striatum. Total huntingtin lowering in the brain has been shown to be well tolerated in adult mice and a total knock down of huntingtin in the striatum and cerebral cortex followed for 14 months also did not lead to obvious pathology.

For translatability to the clinic we have performed a study in transgenic HD minipigs also utilizing intra-cranial administration of AAV5-miHTT. We previously described that treatment was well tolerated and no histological pathology was found. Widespread vector distribution and miHTT expression was found throughout the minipig brain. A clear trend towards dose-dependent reduction of human mutant huntingtin was shown, with up to 85% lowering. The larger size and complexity of the minipig brain, compared to rodents, allows for better translatable of the AAV5-miHTT treatment to the clinic. R6/2 mice express a truncated mutant huntingtin cDNA fragment, resulting in CNS as well as systemic pathology and a greatly reduced life-span. R6/2 mice start dying around 10 weeks of age, with the median death between
14-18 weeks. The initial onset of motor symptoms signals is around 3 weeks of age and mice in this study were injected with AAV5-miHTT at 4 weeks of age, which is during the symptomatic phase. In the current study, all groups treated with AAV5-miHTT (single intra-striatal injection) had an increased survival when compared to the vehicle group. The median survival was significantly improved by 29 days in the high dose group. In a previously reported study with intracerebroventricular infusions of ASO targeting huntingtin mRNA, a 23 day improvement in median survival was shown after daily treatments with a high total dose of ASOs. Another proposed therapeutic for HD, a p75 neurotrophin receptor ligand LM11A-31, demonstrated an increase of mean survival of 17 days in R6/2 mice. Many other studies have not investigated or reported survival or failed to see an improvement on survival after treatment. Since the R6/2 mouse model displays a clear motor phenotype, this has been used a functional outcome measure in many studies. Eight weeks after AAV5-miHTT treatment, the high dose group showed a 56% rotarod improvement compared to control animals. The rotarod test could not be performed at later time points because this would have been too strenuous on the mice. Other studies have also shown rotarod improvement, such as a 30% rotarod improvement one week after neural stem cell transplantation and rotarod improvement in all dose groups 7 to 8 weeks after laquinimod treatment at 12 weeks of age. One of the phenotypes found in several HD mouse models that express a partial human huntingtin fragment is decreased body weight compared. Most studies using R6/2 mice do not find a positive effect on body weight. In the current study, there was a significant beneficial effect on body weight of the R6/2 mice at several timepoints after treatment with AAV5-miHTT. Several different therapies (ASO, laquinimod and LM11A-31) that are currently used in clinical trials have all shown some
survival or behavioral improvement in R6/2 mice, but this was not as strong as in the present study.

Altogether, the data from the current studies corroborate and extend the strong evidence for the therapeutic efficacy of the AAV5-miHTT vector by inducing sustained huntingtin protein lowering in the brain. AAV5-miHTT mediated huntingtin lowering resulted in functional improvement in a severe mouse model for HD. It was shown that even up to 12 months after a single treatment with AAV5-miHTT, significant lowered huntingtin protein levels were observed and without raising any safety concerns. The phenotypical improvement in the R6/2 mice was seen both in a significant increase in median survival and in motor function, demonstrated by Rotarod testing. The sustained human mutant huntingtin lowering, functional improvement, survival benefit and tolerability of AAV5-miHTT, supports further development of the huntingtin-lowering gene therapy and initiation of clinical trials in HD patients in the near future.
Materials and Methods

Animals

All procedures were carried out in accordance with the European Community directive (86/609/EEC) for the care and use of laboratory animals, using protocols approved and monitored by the Animal Experiment Board of Finland. Heterozygous Q175 KI, R6/2 mice and their wild-type littermates were bred by Charles River Labs, Germany and genotyped at weaning. Animals were housed in a temperature-controlled room and maintained on a 12 h day/night cycle. Food and water were available ad libitum.

AAV5-miHTT

AAV5 vector encoding cDNA of the miHTT cassette was produced by a baculovirus-based AAV production system (uniQure, Amsterdam, The Netherlands) as described previously. Expression was driven by a combination of the cytomegalovirus early enhancer element and chicken β-actin promoter and the transcription unit was flanked by two non-coding AAV-derived inverted terminal repeats.

Surgical procedures

Standard aseptic surgical procedures were used to perform the injections. Briefly, mice were anesthetized with 5% isoflurane and placed into a stereotactic frame. During the operation the concentration of anesthetic was reduced to 1.0-1.5%. Two µl of vehicle or AAV5-miHTT was injected into the striatum in both hemispheres (AP = +0.8 mm; ML = ±1.8 mm; DV = -3.0 mm) using a 10-µl Hamilton syringe at a rate of 0.4 µl/min. The needle was left in place for 3 min after surgery, retracted by 1 mm and left for another 3 minutes after which the needle was
completely removed. One hour before and for 48 hrs after surgery mice were administered buprenorphine (Temgesic, 0.03mg/kg, 1ml/kg subcutaneously) for analgesia.

Animal perfusion and tissue collection

All of the Q175 KI and wild type mice and the R6/2 mice that were alive until 28 weeks of age were terminally euthanized at the end of the study. The mice were perfused with ice cold heparinized saline, after the brain was removed, and the right cortex and striatum were collected and frozen in liquid nitrogen and kept at -80 C until further analysis. The left hemisphere of the brain was stored in 4% PFA for further analysis.

Clinical chemistry

Clinical biochemistry panel of markers were analyzed in un-hemolyzed serum for the following parameters: alkaline phosphatase (AFOS), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), Albumin (ALB), gamma-glutamyl transferase (GGT), creatinine (Crea), total protein (Prot), urea (UREA), calcium (Ca), cholesterol (Chol) and inorganic phosphate (Pi).

Quantitative PCR DNA and RNA

Q175 KI mouse and wild type littermate brain samples from the right cortex were used for DNA and RNA isolation and levels were measured by quantitative PCR. Tissue was crushed using the CryoPrep System (Covaris, Woburn, MA) and powder was divided for DNA and RNA analysis. DNA was extracted from pulverized cortex tissue with the DNAeasy 96 Blood and Tissue kit (Qiagen, Germany). Primers specific for the CAG promoter were used to amplify a sequence specific for the transgenes by SYBR Green Fast qPCR (Thermo
Fisher Scientific). The genome copies per µg of genomic DNA input of the samples were calculated by interpolation of a standard line of the expression cassette. To define the background levels of the qPCR a blank sample was subjected to qPCR using the same expression cassette targeting primers.

Total RNA was extracted from crushed cortex tissue and used to determine the level of miHTT (the microRNA expression) and huntingtin mRNA expression (the target). For the miHTT miRNA expression RNA was used to reverse transcribe RNA into cDNA using a gene specific RT-PCR using a stem-loop RT primer specifically targeting the miHTT. Each cDNA sample was subjected to TaqMan qPCR using a primer-probe set specific for miHTT and an internal control miRNA (U6). Mature miHTT levels in the samples were calculated by normalized control miRNA and relative to the vehicle control Q175 KI group. To control for gDNA contamination in the samples an RT- and no template control sample were subjected to qPCR using the same primer-probe set specific for miHTT.

Total RNA was treated with DNase (Thermo Scientific, Waltham, MA) to remove genomic DNA and reverse transcribed into cDNA using a DyNAmo cDNA synthesis kit (Thermofisher Scientific, F-470L) following manufacturer’s instructions. Each cDNA sample were subjected to TaqMan qPCR using a primer-probe set targeting human HTT and an internal control house-keeping gene (GAPDH). Human HTT expression levels were calculated by normalizing to the house-keeping gene and relative to the average of the Q175 KI vehicle control group based on 2^(-ΔΔCt) method. To control for DNase treatment efficacy/efficiency and potential gDNA contamination in the samples a RT-, DNase-, RT-DNase+ and a no template control sample were subjected to qPCR using the same primer-probe set, specific for human HTT.
The RT reaction was performed on a Biometra GMBHT advanced Thermocycler and qPCR runs were performed on an ABI-7500 fast real-time qPCR cycler.

**HTT protein expression in brain tissue homogenates**

Quantification of mutant huntingtin protein in Het Q175 mouse brain samples was performed at Evotec (Hamburg, Germany) using a customized Mesoscale Discovery (MSD) assay. Pulverized brain samples were homogenized in 170 µl ice cold lysis buffer (Dulbecco’s PBS + 0.4 % Triton X-100 + protease- and phosphatase inhibitor) using a FastPrep24 tissue homogenizer. The homogenates were aliquoted, frozen on dry ice and stored at -80°C until further use. With one aliquot the total protein concentration was determined by BCA assay. For the MSD assay, the homogenates were first diluted to 0.2 mg/ml total protein with lysis buffer and afterwards 1:2 with MSD blocking buffer. From this dilution technical triplicates were measured on one assay plate in MSD. The MSD plate was coated with the capture antibody 2B7 (5 µg/ml in MSD coating buffer) and detection of expanded HTT was carried out with sulfo-tag labeled MW1 detection antibody (5 µg/ml in MSD blocking buffer). In parallel with the tissue homogenates, the following recombinant standard protein was used for quantification: human HTT-Q46_GST, 1-548 (EV4238), diluted with lysis buffer and spiked into WT mouse brain homogenate (0.2 mg/ml in MSD blocking buffer). Standard protein was applied at a final assay concentration range between 0.04 and 2100 ng/ml. Finally, 1x MSD read buffer was pipetted to assay plate and the plate was read with Mesoscale Imager SI6000. The standard curve was used to extrapolate the levels of mutant HTT protein, and subsequently corrected by the µg of total protein in the sample.

**Immunohistochemistry**
The left hemisphere of the brain was embedded in paraffin and sections were processed for immunostaining with EM48, an antibody that is specific for aggregated huntingtin.\textsuperscript{35} The brains were cut into 20\,um thick sections on 200\,um interval, mounted on slides and stored at -80\,C until used for immunostaining. First paraffin was removed from the slides with Roti-clear and descending alcohol series. This was followed by incubation with 0.01 M citric acid at 80\,C. Sections were then washed with TBS-T 2\times 5\,min and all sections were then permeabilized and blocked with 3\% bovine serum albumin (BSA) and 10\% normal goat serum (NGS) containing 0.3\% Triton-X-100 in TBS for 30\,min. Sections were washed 3\% BSA for 5\,min, incubated with EM48 primary antibody (Mouse anti-mutant HTT inclusion, mEM48; Millipore, MAB5374, Dilution 1:500) overnight at room temperature before washing them with TBS 3 \times 10\,min. Secondary antibody incubation (biotinylated goat-anti-mouse, Vector BA-9200, dilution 1:500) was done for 2\,h at RT. All sections were then washed with TBS 3 \times 5\,min.

All sections were then incubated with peroxidase avidin/ biotin complex (Vector PK-6100, dilution 1:500) for 2 hours at RT. After washing in PBS 3 \times 5\,minutes, DAB was used as a substrate and the reaction was monitored by visual inspection of the color formation for a few minutes, and then using H\textsubscript{2}O to stop the reaction. Finally, the sections were dehydrated and mounted with Roti-Mount mounting medium (Carl Roth). Representative images of striatum and motor cortex were taken using Axio Imager.M2 microscope (Zeiss, Germany) using an EC Plan-Neofluar 20x objective (N.A. 0.5; W.D. $\infty$) and EC Plan-Neofluar 40x objective (N.A. 0.75; W.D. $\infty$) (Carl Zeiss). Images for EM-48 were analyzed for number of aggregates per area in the cortical and striatal regions. Image analysis was performed using a particle count macro written for ImageJ software. Striatum and motor cortex were delineated manually from each analyzed section. Then the delineated area (total area) was thresholded by visual inspection to highlight
any positive staining found within the ROI. Positively stained aggregates were detected and counted and number of aggregates per area was calculated.

For GFP staining the brains were embedded in paraffin. From these blocks, sections of 4µM were cut with the microtome and mounted on slides. After drying the slides overnight, the sections were dewaxed and rehydrated. Tissue sections were blocked with PBS/5%BSA/5%NGS to prevent background staining, followed by an overnight incubation at 4°C with anti-GFP (ab290) 1:1000. The second day, the sections were with treated with 1% H₂O₂/30% ethanol/PBS to inactivate endogenous peroxidase. Rabbit Envision (Dako) was applied on the sections for 30 minutes. The staining was developed with DAB substrate kit (abcam: ab64238) and images were acquired on a Leica Aperio Versa 8.

**Behavioral analysis**

**Accelerating Rotarod test**

Q175 mice were tested at 3 months of age (pre-treatment baseline) and 3, 6 and 9 months after dosing. The R6/2 mice were tested at 4 weeks of age (pre-treatment baseline) and 4 and 8 weeks after dosing. The rotarod test was performed during the light phase of the animals. Each session included a training trial of 5 min at 4 RPM on the rotarod apparatus (AccuScan Instruments, Columbus, USA). After the training trial the animals were tested for 3 consecutive accelerating trials of 6 min with the speed changing from 0 to 40 RPM over 360 seconds and an inter-trial interval at least 30 min. The latency to fall from the rod was recorded. Mice remaining on the rod for more than 360 seconds were removed and their time scored as 360 sec.

**Open field test**
Q175 mice were tested at 3 months of age (pre-treatment baseline), 3, 6 and 9 months after dosing. The mice were brought to the experimental room for at least 1 h acclimation to the experimental room conditions prior to testing. The open field test was performed during the early nocturnal phase of the animals. Activity chambers (Med Associates Inc, St Albans, VT; 27 x 27 x 20.3 cm) were equipped with infrared (IR) beams. Mice were placed in the center of the chamber and their behavior was recorded for 30 min in 5-minute bins. Quantitative analysis was performed on the following five dependent measures: total locomotion, locomotion in the center of the open field, rearing rate in the center, total rearing frequency and velocity. Animals were tested at low-stress conditions where the light was lowered to approximately 10-30 lux of red light.

**MotoRater**

The fine motor skills and gait of Q175 mice were measured in the MotoRater (TSE Systems, Homburg, Germany) at 6 and 9 months after dosing, during the light phase. Prior to the test, the mice were shaved under light isoflurane anesthesia, and the essential body points marked for tracking.

The data regarding gait performance was captured using a camera with a speed of 300 frames / second, imaging the gait from three different views (below and both sides). The captured videos of each mouse were first converted to SimiMotion software to track the marked points of body from the video, thereby attaining the raw data of analysis. The raw data thus comprised of the movements of different body points in coordinates related to the ground and each of the three dimensions.
Different gait patterns and movements were analyzed using a custom-made automated analysis system. The data was then analyzed regarding several specific clusters of parameters and all fine motor data were analyzed for 97 distinctive parameters. The parameter data were further processed using principal component analysis (PCA).

**Video analysis**

This analysis was done on R6/2 mice at 12 weeks of age, during the light phase. Per group 5 animals were filmed to observe clasping behavior. The mice were picked up by the tail for approximately 30 seconds to induce the clasping behavior. Two individual observers scored the videos for the time it took the mice to touch the hindlegs together and the time it took until the hindlegs were fully clasping.

**Statistics**

Mean values were used for statistical analyses. Data are expressed as means +/- SEM. Student t test was used for statistical comparison between two groups.

For comparisons of more than two groups, one-way analysis of variance (ANOVA) was used followed by the Dunnetts/Tukey multiple comparison post-hoc test (Prism; GraphPad Software, San Diego, CA). For both tests, p < 0.05 was considered a statistically significant difference.
Disclosure/Conflict of interest

M.D.H. and H.P. have close affiliations with uniQure.

Acknowledgments

The authors thank Jacek Lubelski, Erich Ehlert, Tamar Grevelink, Lisanne Schulte, Mark van Veen, Maroeska Oudshoorn, Sumiati Baatje and Valerie Sier-Ferreira (uniQure) for technical support. The authors also thank Eileen Sawyer, Wim Hermens (uniQure) and Olivier ter Brake (EP&C) for critically reviewing the manuscript. The authors declare no conflict of interest.

Author Contributions

References


Figure Legends

**Figure 1.** AAV5-miHTT administration in mouse brain using a single bilateral intra-striatal stereotactic injection. (A) Experimental setup (n = 8 per group) showing wild type and the Q175 KI mice used in the study with the different dose groups (B) Experimental setup, behavioral tests were performed to assess motor skills at several time points as indicated. Day of dosing (at 3 months of age) and necropsy (at 15 months of age) are also indicated, (C) Brain sampling overview. Striatum and cortex were dissected from the rest of the brain. The cortex was cryo-pulverized, and the powdered tissue was divided into three parts, one for vector DNA analysis, one for RNA analysis and one for huntingtin protein analysis. The striatum was only used for huntingtin protein analysis.

**Figure 2.** AAV5-miHTT vector DNA distribution and miHTT expression 12 months after striatal injection in Q175 KI brain. (A) Vector DNA copies (gc per µg total genomic DNA) in the cortex collected from the right hemisphere of each animal (also shows mean±SEM). (B) Mature miHTT expression in cortex from the right hemisphere were determined by custom TaqMan RT-qPCR, values are represented as relative expression for each animal (also shows mean±SEM).

**Figure 3.** AAV5-miHTT treatment demonstrates human mutant huntingtin target engagement in Q175 KI mouse striatum and cortex. (A) MSD quantification of soluble human mutant huntingtin protein levels in striatum and cortex from the right hemisphere (mean±SEM). Data were evaluated using ordinary one-way ANOVA, **p<0.01, ***p<0.001. (B) Immunohistochemistry staining of striatum and cortex from the left hemisphere using the EM48 antibody which is specific for mutant huntingtin protein. (C) Quantification of mEM48 staining
of striatum and cortex (mean±SEM). Data were evaluated using ordinary one-way ANOVA, *p<0.05.

**Figure 4.** AAV5-miHTT administration in mouse brain using a single bilateral intra-striatal stereotactic injection. (A) Experimental setup showing wild type and R6/2 mice used in the study with the different dose groups (B) Experimental setup, n=20 per group. Behavioral tests were performed to assess motor skills at several time points as indicated. Arrows indicate dosing (at 4 weeks of age) and scheduled necropsy of surviving mice (at 28 weeks of age).

**Figure 5.** Bodyweight development over time in R6/2 mice. AAV5-miHTT treatment at the high dose shows significant higher body weight in R6/2 mice when compared to untreated R6/2 mice at several time points between 10 and 21 weeks of age (shown as asterisk in graph).

**Figure 6.** AAV5-miHTT treatment shows motor coordination improvement in R6/2 mice. (A) Untreated R6/2 mice had a significantly lower latency to fall on the rotarod when compared to WT littermates. An increase of latency to fall of 47% was found in R6/2 mice treated with a high dose of AAV5-miHTT (mean±SEM, p<0.05) (B) Videos from 5 animals per group were scored for the time to touch and time to clasp hindlegs. Even though there was no significant effect found, both parameters show a trend toward the WT animals after AAV5-miHTT treatment (mean±SEM).

**Figure 7.** Kaplan-Meier curve showing survival of untreated and AAV5-miHTT treated R6/2 mice. Median survival was increased significantly in the low and high dose groups (26.5 and 29 days, respectively; p<0.05).
**A**

<table>
<thead>
<tr>
<th>Group and strain (n=8)</th>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – Wild type</td>
<td>Vehicle</td>
<td>Vehicle AAVS-miHHT AAVS-miHHT AAVS-miHHT</td>
</tr>
<tr>
<td>2 – Q175 HET KI</td>
<td>Vehicle</td>
<td>Low 1x Mid 5x High 25x</td>
</tr>
<tr>
<td>3 – Q175 HET KI</td>
<td>AAVS-miHHT</td>
<td>AAVS-miHHT</td>
</tr>
<tr>
<td>4 – Q175 HET KI</td>
<td>AAVS-miHHT</td>
<td>AAVS-miHHT</td>
</tr>
<tr>
<td>5 – Q175 HET KI</td>
<td>AAVS-miHHT</td>
<td>AAVS-miHHT</td>
</tr>
</tbody>
</table>

---

**B**

- **AAV5-miHHT administration**
  - Age (months)
  - Open field Rotarod
  - Open field MotorRaiser
  - Histopathology
  - Clinical chemistry
  - Biomolecular analysis
  - Necropsy

---

**C**

- Brain
  - Cortex
  - Striatum
  - Huntingtin protein
  - Huntingtin protein
  - MDA and HTT mRNA
  - Vector DNA

---

**ACCEPTED MANUSCRIPT**

---

**ACCEPTED MANUSCRIPT**

---

**ACCEPTED MANUSCRIPT**
A Vector genome copies right cortex

Vector genome copies per μg DNA

Vehicle Low Mid High

AAV5-miHTT

LLOQ

B mHTT levels right cortex

mHTT levels (relative to vehicle)

0.1 Vehicle Low Mid High

AAV5-miHTT

LLOQ
### A

<table>
<thead>
<tr>
<th>Group and strain</th>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – wild type</td>
<td>Vehicle</td>
<td>-</td>
</tr>
<tr>
<td>2 – R6/2</td>
<td>Vehicle</td>
<td>-</td>
</tr>
<tr>
<td>3 – R6/2</td>
<td>AAV5-miHTT</td>
<td>Low 1x</td>
</tr>
<tr>
<td>4 – R6/2</td>
<td>AAV5-miHTT</td>
<td>Mid 5x</td>
</tr>
<tr>
<td>5 – R6/2</td>
<td>AAV5-miHTT</td>
<td>High 25x</td>
</tr>
</tbody>
</table>

### B

- **AAV5-miHTT administration**
- Age (weeks) 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
- Survival
  - Rotarod
  - Rotarod
  - Rotarod
  - Clasping
AAV5-miHTT