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Developing genetic therapies for polyglutamine disorders

Evers, M.M.

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Author: Evers, Melvin Maurice
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Melvin M. Evers [1]
Barry A. Pepers [1]
Judith C.T. van Deutekom [2]
Susan A.M. Mulders [2]
Johan T. den Dunnen [1,3,4]
Annemieke Aartsma-Rus [1]
Gert-Jan B. van Ommen [1]
Willeke M.C. van Roon-Mom [1]

[1] Department of Human Genetics, Leiden University Medical Center
[2] Prosensa Therapeutics B.V., Leiden
[3] Leiden Genome Technology Center, Leiden University Medical Center
[4] Department of Clinical Genetics, Leiden University Medical Center

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Targeting several CAG expansion diseases by a single antisense oligonucleotide

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3.1. Abstract

To date there are 9 known diseases caused by an expanded polyglutamine repeat, with the most prevalent being Huntington's disease. Huntington's disease is a progressive autosomal dominant neurodegenerative disorder for which currently no therapy is available. It is caused by a CAG repeat expansion in the *HTT* gene, which results in an expansion of a glutamine stretch at the N-terminal end of the huntingtin protein. This polyglutamine expansion plays a central role in the disease and results in the accumulation of cytoplasmic and nuclear aggregates. Here, we make use of modified 2'-O-methyl phosphorothioate $(CUG)_n$ triplet-repeat antisense oligonucleotides to effectively reduce mutant huntingtin transcript and protein levels in patient-derived Huntington's disease fibroblasts and lymphoblasts. The most effective antisense oligonucleotide, $(CUG)_7$, also reduced mutant ataxin-1 and ataxin-3 mRNA levels in spinocerebellar ataxia 1 and 3, respectively, and atrophin-1 in dentatorubral-pallidoluysian atrophy patient derived fibroblasts. This antisense oligonucleotide is not only a promising therapeutic tool to reduce mutant huntingtin levels in Huntington's disease but our results in spinocerebellar ataxia and dentatorubral-pallidoluysian atrophy cells suggest that this could also be applicable to other polyglutamine expansion disorders as well.

3.2. Introduction

Polyglutamine (polyQ) diseases are a group of disorders caused by CAG triplet repeat expansions in the coding region of the genome. The disease causing proteins in these polyQ diseases are very different, but in each case the expanded stretch of glutamines results in a toxic-gain-of function of the protein and this leads to neurodegeneration. To date, a total of 9 polyQ disorders have been described: dentatorubral-pallidoluysian atrophy (DRPLA), Huntington's disease (HD), spinal bulbar muscular atrophy (SBMA), and spinocerebellar ataxias (SCA1, 2, 3, 6, 7, and 17) (Cummings and Zoghbi, 2000; Nakamura *et al.*, 2001). Of these polyQ disorders, HD and SCA3 have the highest prevalence worldwide (Bauer and Nukina, 2009). The expanded repeats in these polyQ diseases are unstable resulting in anticipation; a more severe and earlier onset of disease in following generations (Ranen *et al.*, 1995). There is an inverse correlation of disease onset and polyQ length in the protein; the longer the CAG repeat, the earlier the age of onset of the disease (Cummings and Zoghbi, 2000). Protein aggregates are found in the nucleus and cytoplasm of cells, indicating that protein misfolding is a common feature of these disorders. Currently no treatment is available to delay onset or even slow progression of polyQ diseases.

In HD, the expanded CAG repeat is located in the first exon of the *HTT* gene on chromosome 4p16. The expanded CAG transcript is translated into a mutant huntingtin (htt) protein with an expanded polyQ tract at the N-terminus. Patients with 40 or more CAG repeats will develop the disease, whereas people with 35 to 39 repeats show reduced penetrance (Mcneil *et al.*, 1997). The disease is characterized by motor, psychiatric and cognitive impairments and the typical age of onset lies between 30 and 50 years (Andrew *et al.*, 1993). The major neuropathology occurs in the striatum but degeneration is seen throughout the brain when the disease progresses. Various other proteins have been found to co-localize with htt aggregates, i.e. TATA box binding protein (TBP), CREB binding protein (CBP) and several molecular chaperones (Huang *et al.*, 1998; Steffan *et al.*, 2000; Muchowski *et al.*, 2002; Roon-Mom *et al.*, 2002). When the mutation for HD was found, htt was a protein of unknown function but extensive research over the past decade has revealed numerous functions for htt. Also many affected cellular processes have been identified in HD, such as transcriptional de-regulation, mitochondrial dysfunction, and impaired vesicle transport (Bauer and Nukina, 2009; Ross and Tabrizi, 2011).

SCAs are genetically and clinically distinct autosomal dominant CAG-expansion diseases, numbered by the order of gene description. Patients with SCA exhibit cerebellar degeneration resulting in ataxia and oculomotor deficits, often followed by general brain atrophy (Manto, 2005; Schols *et al.*, 2004). The first SCA identified, SCA1, is caused by a CAG repeat expansion of 41 or more in exon 8 of the *ATXN1* gene (Bauer and Nukina, 2009). *ATXN1* is translated into the 98 kDa protein ataxin-1, which is involved in transcriptional regulation and RNA metabolism (Matilla-Duenas *et al.*, 2008). Mutated ataxin-1, by entering the nucleus, causes cellular dysfunction (Klement *et al.*, 1998). In SCA3, the expanded CAG repeat is located in exon 10 of the *ATXN3* gene which is translated into mutant ataxin-3 (Kawaguchi *et al.*, 1994).

Patients develop the disease when the number of CAGs exceed 51, while there is reduced penetrance when the number of repeats is between 45 and 51 (Padiath *et al.*, 2005). The 42 kDa ataxin-3 protein is suggested to be involved in proteasomal degradation and transport of ubiquitinated proteins (Riess *et al.*, 2008). DRPLA is a rare autosomal dominant disorder, characterized by dementia, ataxia, chorea, myoclonic epilepsy, and psychiatric disturbances. The disease is caused by a CAG repeat expansion in exon 5 of the *ATN1* gene, which encodes the 200 kDa atrophin-1 protein. Atrophin-1 is a known transcriptional co-regulator although its exact function is not well understood (Shen and Peterson, 2009). Patients with a repeat of 49 or more glutamines will develop the disease (Nagafuchi *et al.*, 1994).

Most therapeutic strategies under investigation for polyQ disorders are aimed at counteracting one of the many cellular processes that are altered due to expression of the mutant protein. For instance, in all of these neurodegenerative diseases the formation of fragmented protein products by proteolytic cleavage is an important step in the pathogenic process (Bauer and Nukina, 2009). It has been shown that altering proteolysis of the mutant htt protein can be beneficial, as an HD mouse model lacking the caspase 6 cleavage site had reduced neuronal dysfunction and neurodegeneration (Graham *et al.*, 2006). Reducing mutant polyQ protein levels and thereby inhibiting all downstream toxic effects would be much more effective than targeting a single cellular process. One way to achieve this would be to enhance the degradation of mutant polyQ proteins through activation of the proteasome (Seo *et al.*, 2007) or through upregulation of the autophagic pathway (Metcalf *et al.*, 2010). Another strategy would be to inhibit the formation of mutant polyQ proteins by gene silencing or transcript degradation (Scholefield and Wood, 2010). RNAi is increasingly used as a potential therapeutic tool to reduce expression of target transcripts (Rao *et al.*, 2009). RNAi is an endogenous cellular defense mechanism against exogenous viral components and is also involved in transcriptional regulation (Ding and Voinnet, 2007). Specific knock down of target sequences is achieved by introducing exogenously modified oligonucleotides (e.g. short hairpin RNA (shRNA) and short interfering RNA (siRNA)) that bind to the target transcript, which is subsequently degraded or its translation blocked. Recently an siRNA targeting both normal and mutant htt was found to be well-tolerated in wild-type rats (Drouet *et al.*, 2009). However, endogenous htt expression is important for normal cellular function, as underlined by the finding that conditional knockout of murine htt in forebrain and testis resulted in loss of function and progressive neurodegeneration (Dragatsis *et al.*, 2000). Total loss of the endogenous htt homolog in a Drosophila HD model expressing the human first exon of the *HTT* gene with 93 Qs enhanced the HD pathogenesis (Zhang *et al.*, 2009a). These studies show that a specific reduction of mutant htt levels, leaving as much wild-type htt protein as possible, would be the optimal outcome of a therapy aimed at htt knockdown. Specific reduction of the mutant htt transcript was shown by allele-specific siRNAs directed against a single nucleotide polymorphism (SNP) in htt exon 50 (van Bilsen *et al.*, 2008). In a recent study on the cleavage of triplet repeat hairpins by ribonuclease dicer it was shown that an siRNA with 7 consecutive CUG nucleotides specifically reduced the expression of the mutant htt transcript containing 44 CAG repeats in HD human fibroblasts (Krol *et al.*, 2007). Although off-target effects and

interference with endogenous RNAi processes remains to be assessed (McBride *et al.*, 2008), these results are encouraging.

Another RNA based therapy approach to knock down gene or protein expression is the use of single stranded antisense oligonucleotides (AON). One of the most promising examples of AON treatment in a neurodegenerative disease is aimed at amyotrophic lateral sclerosis (ALS). In ~2% of ALS patients, the disease is caused by a mutation in superoxide dismutase 1 (SOD1) (Robberecht, 2000). Continuous intraventricular infusion of AONs successfully down regulated SOD1 mRNA and protein levels in the brain and significantly slowed disease progression in an animal model of ALS (Smith *et al.*, 2006). A clinical trial is currently ongoing in ALS patients with SOD1 mutations and results are expected this year (ClinicalTrials.gov, 2009).

For glutamine-expansion disorders, peptide nucleic acid (PNA) and locked nucleic acid (LNA) antisense oligomers targeting CAG repeats have been used to reduce expanded HD and SCA3 transcripts *in vitro* (Hu *et al.*, 2009a; Hu *et al.*, 2009b; Gagnon *et al.*, 2010; Hu *et al.*, 2011). However, although PNA transfection efficiently reduced mutant protein levels with very long glutamine expansions, the reductions on polyQ lengths that occur most frequently in the HD patient population were less pronounced (Hu *et al.*, 2009a; Hu *et al.*, 2009b). In the current we make use of 2'-O-methyl (2OMe) modified RNA AONs with a phosphorothioate (PS) backbone carrying different CUG numbers. We examine the effect of (CUG)n AONs on mRNA level in cell lines derived from HD, SCA1, SCA3, and DRPLA patients with CAG expansions that occur most frequently in the patient population. A significant reduction in expanded transcript levels was found in patient derived fibroblast from HD, SCA1, SCA3, and DRPLA. Furthermore a significant reduction of mutant htt protein was seen in the HD cells. For htt, a reduction in wild-type htt transcript levels was observed as well, but this reduction was less pronounced than for the mutant transcript. Lowering the AON concentration increased the specificity for the mutant transcript. These results show that one single antisense oligonucleotide could be a promising therapeutic treatment for all polyQ disorders.

3.3. Materials & Methods

Cell culture and transfection

Patient derived fibroblasts from HD (GM04022), SCA3 (GM06151), SCA1 (GM06927), and DRPLA (GM13716) (purchased from Coriell Cell Repositories, Camden, USA); and control fibroblasts FLB73 (kind gift from Dr. M.P.G. Vreeswijk, LUMC) were cultured at 37°C and 5% CO_2 in Minimal Essential Medium (Gibco Invitrogen, Carlsbad, USA) with 15% heat inactivated fetal bovine serum (Clontech, Palo Alto USA), 1% Glutamax (Gibco) and 100 U/ml penicillin/ streptomycin (Gibco). Human Epstein Barr Virus transformed lymphoblasts HL2.42 and HL2.93 were a kind gift from Prof. E. Bakker (Laboratory of Diagnostic Genome Analysis (LDGA), LUMC). Cells were cultured at 37°C and 5% CO_2 in RPMI 1640 medium (Gibco), containing 15% FBS, 1% glutamax and 100 U/ml P/S.

AON transfection was performed with 3.3 µl ExGen 500 polyethylenimine (PEI) (MBI Fermentas, Vilnius, Lithuania) per µg AON. AON and PEI were diluted in 150 mM NaCl to a total volume of 100 µl and mixtures were prepared according to the manufacturer's instruction. Four different transfection conditions were used: 1) transfection with 1-100nM $(CUG)_7$, 100nM $(CUG)_3$, 100nM $(CUG)_{12}$, 2) transfection with 10-100nM h40AON2 directed against exon 40 of the *DMD* gene (5'- UCC UUU CAU CUC UGG GCU C -3') (Control AON) (Aartsma-Rus *et al.*, 2002), 3) transfection without AON (Mock II), and 4) NaCl only (Mock I). Mixtures were added to a total volume of 2 ml of medium with 5% FBS. Four hours after transfection, medium was replaced with fresh medium and a second identical transfection was performed 24 hours after the first transfection. All AONs consist of 2'-O-methyl RNA and contain a full-length phosphorothioate backbone (Prosensa B.V. Leiden, the Netherlands).

RNA Isolation and RT-PCR

Forty eight hours after the first transfection cells were harvested by trypsinization and washed twice with Hanks buffered salt solution (HBSS) (Gibco). Total RNA was isolated from the cells using an RNeasy Mini Kit (QIAgen, Venlo, The Netherlands), with an on-column DNase treatment for approximately 30 minutes. RNA was eluted in 50 µl elution buffer and cDNA was synthesized from total RNA using the Transcriptor First Strand cDNA Synthesis Kit with Random Hexamer primers at 65°C (Roche, Mannheim, Germany).

PCR was performed using 1 µl cDNA, 10x PCR buffer with 1.5 mM $MgCl_2$ (Roche), 2mM dNTPs, 10 pmol forward primer, 10 pmol reverse primer, 1U FastStart Taq DNA Polymerase (Roche), 1M Betaine (Sigma-Aldrich, St. Louis, USA), and PCR grade water to a final volume of 20 µl. PCR was performed with primers for HTT, ATXN1, ATXN3, and ATN1 (all flanking the CAG repeat), ACTB, and RPL22 (for sequences, see **Table S1**). The PCR program started with a 4 min initial denaturation at 95°C, followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 60°C (56°C for ATXN3), 45 sec elongation at 72°C, after which a final elongation step was performed at 72°C for 7 min.

Lab-on-a-Chip was performed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), using the Agilent DNA 1000 Kit. Expression levels were normalized for β-actin levels and relative to transcript levels without transfection (Mock I). The relative mutant transcript levels were analyzed using a paired two-sided Student t test. Differences were considered significant when $P < 0.05$.

qPCR, Calculations and Sequencing. The qPCR was performed using 1 µl of 5x diluted cDNA, 2x FastStart Universal SYBR Green Master mix (Roche), 2.5 pmol forward primer, 2.5 pmol reverse primer and PCR grade water to a total volume of 10 µl. Primer pairs for 6 transcripts containing long uninterrupted CAG repeats were selected for qPCR by BLAST analysis and ACTB and RPL22 were used as reference genes. (For primer list, see **Table S1**). The qPCR was performed using the LightCycler 480 (Roche). Initial denaturation was 10 min. at 95°C, followed by 45 cycles of 10 sec. denaturation at 95°C, 30 sec. annealing at 59°C and 20 sec. elongation at 72°C. The final elongation was performed 5 min. at 72°C. Next, we performed a melting curve analysis of all samples from 60°C to 98°C with a ramp rate of 0.02°C per sec.

Relative expression of the transcript levels was calculated as described previously (Pfaffl, 2001). All samples were run in triplicate on a plate and two independent experiments were performed for each sample. On all plates both reference genes were included to correct for inter-plate variance.

Primer efficiencies were determined using LinRegPCR v11.1 (Ruijter *et al.*, 2009) with the raw data amplification curves as input and Mock II was used as reference. Values from the mock water transfected cells (Mock I) were set on 100%. The relative transcript levels were analyzed using a paired two-sided Student t test. Differences between groups were considered significant when $P < 0.05$.

CAG repeats of the CAG enclosing transcripts were amplified using primers flanking the CAG repeat (see **Table S1**). PCR products were loaded on an agarose gel and bands were extracted using the QIAquick Gel Extraction Kit (QIAgen). The purified products were sequenced by Sanger sequencing, using the Applied Biosystems 96-capillary 3730XL system (Life Technologies Corporation, Carlsbad, USA) with the Applied Biosystems BigDyeTerminator v3.1 kit.

Protein isolation and Western blotting

Cells were detached from the culture surface with a 0.5% Trypsin/EDTA solution. After washing twice with 1x HBSS, cells were resuspended in 200 µl ice cold lysis buffer, containing 1x PBS, 0.4% Triton-X100, and 1 tablet Complete mini protease inhibitor EDTA free (Roche) per 10 ml buffer. Next, samples were sonicated 3 times for 5 seconds using ultrasound with an amplitude of 60 at 4°C. After incubation in a head-over-head rotor at 4°C for 1 hour, the extract was centrifuged for 15 min at 10,000g and 4°C and supernatant was isolated. Protein concentrations were determined by the bicinchoninic acid kit (Thermo Fisher Scientific, Waltham, USA) using Bovine Serum Albumin as a standard. Samples were snap frozen and stored at -80°C.

Protein extracts were separated by SDS-PAGE, with 4-15% acryl/bisacrylamide 1:37.5 separating gels and 30 µg (human fibroblasts) of protein lysate loaded. For each sample the Spectra Multicolor High Range Protein Ladder (Fermentas) was used as a marker. Electrophoresis was performed for 30 min at 100V through the stacking gel and 5 hours at 150V through the running gel. Gels were blotted onto a polyvinylidene fluoride (PVDF) membrane for 3 hours at 300mA. Membranes were blocked with 1x Tris Buffered Saline + 0.5% Tween 20 (TBST) containing 5% non-fat milk powder (Profitar Plus, Nutricia, Zoetermeer, the Netherlands). The antibodies used for detection were mouse 4C8 for htt (Eurogentec, Liege, Belgium) dilution 1:1000, mouse 1C2 specific for expanded poly glutamine stretches (Eurogentec) dilution 1:500, mouse ataxin-3 (Eurogentec) 1:1000, rabbit TBP (Santa Cruz Biotechnology, USA) 1:1000, and mouse β-actin, diluted 1:5000. Secondary antibodies were goat α-mouse-horseradish peroxidase (Santa Cruz) and goat α-rabbit-horseradish peroxidase (Santa Cruz), both diluted 1:10.000 in 1x TBST. Horseradish peroxidase was activated by ECL+ reagent (GE Healthcare, Buckinghamshire, United Kingdom) to visualize positive staining on film.

Protein bands were quantified using ImageJ software. The percentage of inhibition was calculated as a relative value to a non-treated control sample and was normalized using β-actin.

3.4. Results

$(CUG)_7$ AON shows most pronounced reduction of HTT transcript levels

Patient-derived human fibroblasts were transfected with AONs with 3, 7 and 12 consecutive CUGs ($(CUG)_3$, $(CUG)_7$, and $(CUG)_{12}$, respectively) and total RNA was isolated after 48 hours. In the *HTT* gene the glutamine repeat consists of a CAG stretch, followed by one CAA and a final CAG triplet. The HD cell line GM04022 contained a $(CAG)_n$ CAA CAG repeat with n = 18 and 44. As a control fibroblasts cell line FLB73 was used where n = 17 and 21. To avoid influences of CAG repeat length, reductions in total HTT mRNA levels were measured by quantitative PCR (qPCR) with primers within the CAG containing exon but amplifying a transcript fragment upstream of the repeat (**Table S1**). The most significant reduction in total HTT transcript of 81% (± 4%) in the HD and 76% (± 4%) in the control fibroblasts was found after $(CUG)_7$ treatment (**Figure 1**). $(CUG)_{12}$ transfection resulted in a significant reduction of total HTT transcript of 78% (± 5%) in the HD and 61% (± 18%) in the control cell line. The (CUG)3 did not show significant reduction of HTT mRNA levels. The $(CUG)_7$ AON was selected for further testing since it was the shortest AON resulting in the most significant reduction in HTT mRNA levels.

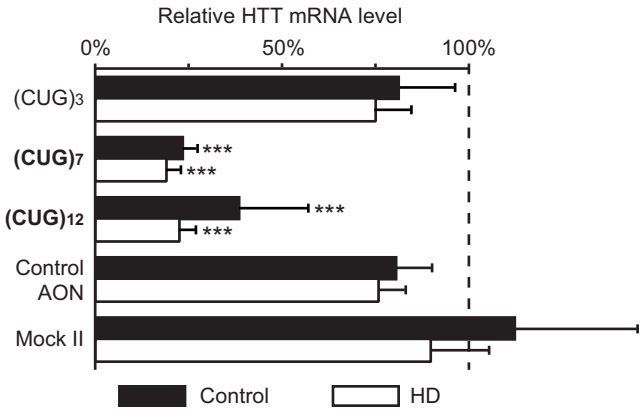

Figure 1. Number of CUGs of AON influences the reduction of HTT transcript levels. Total RNA was isolated 48 hours after transfection. Quantitative RT-PCR was used to measure HTT mRNA levels in control and HD fibroblasts after treatment with 100nM $(CUG)_3$, $(CUG)_7$, $(CUG)_{12}$ AON, 100nM non-htt specific h40AON2 (Control AON), transfection agent only (Mock II), or non-transfected cells (Mock I, not included in this figure). ACTB and RPL22 are used as reference genes. The expression level of Mock I transfections are set to 100%. For all transfections n = 6 and *** $P < 0.001$.

Reduction of mutant HTT mRNA levels in HD cells after $(CUG)_7$ treatment

Since regular htt expression is important for normal cellular function, our approach is to lower mutant htt protein levels, while maintaining sufficient levels of normal protein. To examine the effect of $(CUG)_7$ treatment on both HTT transcripts an allele-specific PCR with primers flanking the CAG repeat was performed in quadruplo (**Figure 2a**). The mutant transcript was decreased by 83% (± 13%, measured by Lab-on-a-Chip analysis) in $(CUG)_7$ treated cells compared to controls, while normal transcript was reduced to a lesser extent with 43% (± 32%) (**Figure 2b**). Treatment of the control cell line with $(CUG)_7$ showed a reduction for both alleles of 21% (± 38%) and 40% (± 38%) respectively.

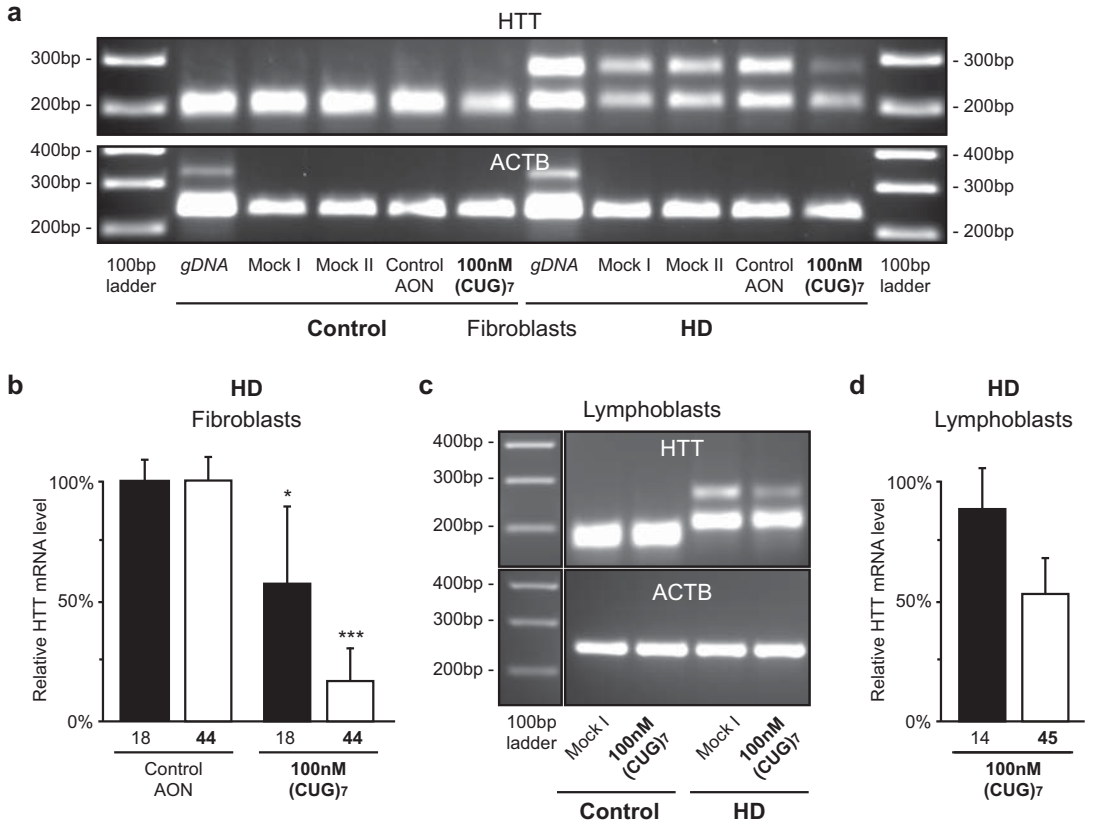

Figure 2. Effect of $(CUG)_7$ AON on HTT mRNA levels in HD patient derived cell lines 48 hours after transfection. Cells were transfected with 100nM $(CUG)_7$, non-htt specific h40AON2 (Control AON), transfection agent only (Mock II), or non-transfected cells (Mock I). **(a)** Agarose gel analysis of the HTT transcript with primers flanking the CAG repeat of control (FLB73) and HD (GM04022) fibroblasts treated with various AONs. Transfection with $(CUG)_7$ shows a decrease of the upper band, representing the transcript from the mutant allele. The lower band, representing the normal HTT transcript, is also reduced, but to a lesser extent. Control cells treated with $(CUG)_7$ only show a slight reduction compared to the control transfections. PCR products with primers for ACTB were used as loading control. gDNA was taken along to control for the PCR reaction over the CAG repeat. **(b)** Lab-on-a-Chip analysis of HTT transcripts after $(CUG)_7$ treatment in a HD fibroblast cell line. The mutant transcript, with 44 CAGs, is significantly reduced by 83% after $(CUG)_7$ treatment, compared to transfection controls. The normal HTT transcript with 18 CAGs is reduced by 43%. Expression levels are corrected for loading differences with ACTB. The mRNA level of the Mock I transfection was set on 100% (* $P < 0.05$, *** $P < 0.001$, n = 4). **(c)** Agarose gel analysis of HTT transcripts after $(CUG)_7$ treatment in EBV transformed control and HD human lymphoblasts. After transfection with $(CUG)_7$ the mutant HTT transcript with 45 CAGs is decreased compared to the Mock transfection. No changes in intensity of the HTT transcripts from the control lymphoblasts are seen after $(CUG)_7$ treatment. **(d)** Lab-on-a-Chip analysis of HTT transcripts after PS57 treatment of human HD lymphoblasts. Mutant HTT transcript is reduced by 46% after $(CUG)_7$ treatment, whereas the normal HTT allele shows an 11% reduction. (n = 2)

We repeated this experiment in duplo in patient-derived Epstein Barr Virus transformed control and HD lymphoblasts (**Figure 2c**). $(CUG)_7$ transfection of the HD cell line gave a reduction of the mutant transcript of 53% (± 10%), while only a small decrease of 22% (± 11%) for the normal transcript was detected (**Figure 2d**). No apparent reduction in the control cell line was found (data not shown).

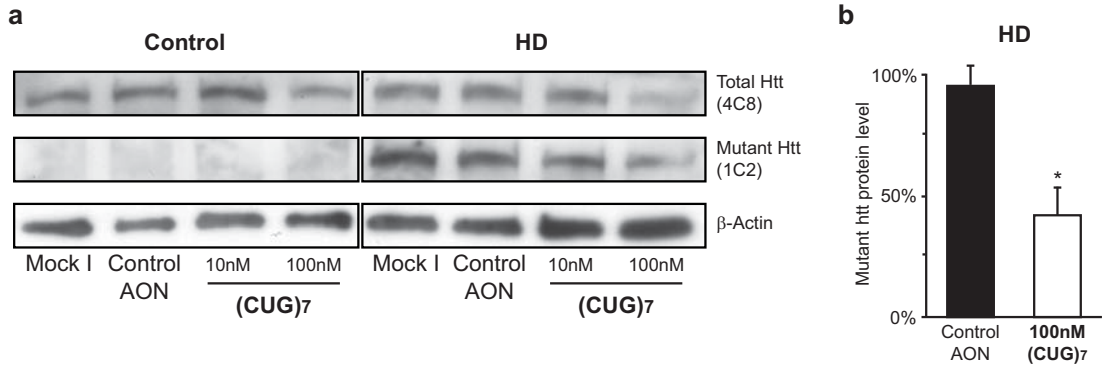

Figure 3. $(CUG)_7$ AON reduces mutant htt protein levels in HD patient fibroblast cell lines. Cells were transfected with 10nM and 100nM $(CUG)_7$, non-htt specific h40AON2 (Control AON), or non-transfected cells (Mock I). **(a)** Western blot of control (FLB73) and HD (GM04022) fibroblasts treated with $(CUG)_7$ and controls. Total (4C8) and mutant (1C2) htt protein expression is reduced 72 hours after treatment with $(CUG)_7$. No mutant htt could be detected in the control fibroblasts with 1C2. β-actin is used as loading control. **(b)** Mutant htt protein levels in HD (GM04022) fibroblasts after 100nM $(CUG)_7$ transfection were quantified by ImageJ software. A significant reduction of 58% of mutant htt protein was seen after $(CUG)_7$ transfection as compared to control transfections (* $P < 0.05$, n = 2). Mutant protein levels of Mock I transfection were set to 100%.

Reduction of mutant htt protein levels in a HD cell line after $(CUG)_7$ treatment

Since mRNA levels of the HTT transcript were substantially reduced after treatment with $(CUG)_7$, in both experiments, we investigated htt protein levels (**Figure 3a**). Antibody 4C8 can be used to detect total htt protein (Trottier *et al.*, 1995a), while antibody 1C2 specifically recognizes the expanded polyQ tract (Trottier *et al.*, 1995b). Patient-derived human fibroblasts were transfected and protein isolated (see Materials and Methods). 96 hours after first treatment of HD fibroblasts with 100nM $(CUG)_7$ 4C8 antibody showed a clear reduction of 54% (± 34%) in htt protein level, while a less pronounced reduction of 16% (± 28%) was observed in the control fibroblasts (**Figure 3a** and data not shown). With 1C2 antibody a significant reduction of 58% (± 16%) of mutant htt protein was seen in the HD fibroblasts following 100nM $(CUG)_7$ treatment (**Figure 3b**). Thus, reduction of mutant htt protein was more pronounced than normal htt.

$(CUG)_7$ AON efficiency is concentration dependent

To test if $(CUG)_7$ AON concentration is related to efficacy, various AON concentrations were used to transfect HD and control fibroblasts. Lab-on-a-Chip analysis (**Figure 4a and b**) showed a reduction of mutant HTT with an IC50 value between 2.5nM and 5nM (**Figure 4b**). At 10nM $(CUG)_7$ the mRNA expression of mutant HTT was reduced by 89% (± 5%), whereas normal HTT transcript was reduced by 38% (± 9%) in the HD fibroblasts. HTT mRNA reduction was less pronounced for both alleles (16% (± 6%) and 36% (± 5%)) in the control cells, suggesting that at lower concentrations the $(CUG)_7$ AON is more specific at reducing HTT transcripts with expanded CAG repeats (**Figure 4a**).

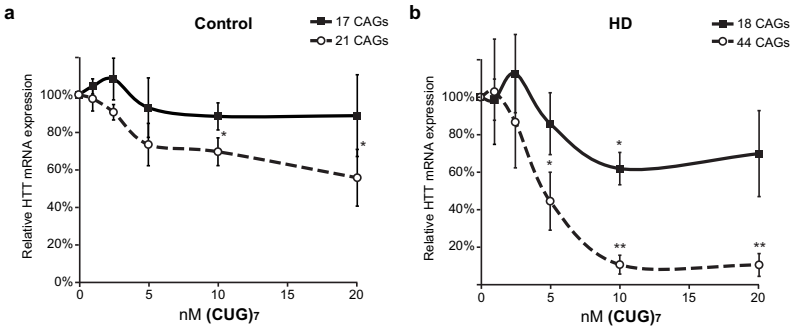

Figure 4. Effect of various $(CUG)_7$ AON concentrations on HTT mRNA expression. Cells were transfected with 1-20nM $(CUG)_7$. PCR products with primers flanking the CAG repeat of HTT were quantified by Lab on a Chip. **(a)** In the control cell line (FLB73) both alleles (17 and 21 CAGs) show a comparable concentration dependent reduction of HTT mRNA quantification after $(CUG)_7$ transfection. **(b)** In HD fibroblasts (GM04022) the mutant transcript, with 44 CAGs, shows a strong reduction of mutant HTT mRNA expression with increasing $(CUG)_7$ AON concentrations, whereas the normal HTT transcript with 18 CAGs is reduced to a lesser degree. Expression levels are corrected for loading differences with ACTB and mRNA levels of the Mock I transfections were set on 100% (* $P < 0.05$, ** $P < 0.01$, n = 4).

AON directed against the CAG repeat reduces mutant ataxin-3 levels

Since CAG repeat expansions are a hallmark of several neurodegenerative disorders, we tested the molecular efficacy of our AON approach to reduce the expression of other genes as well. SCA3 patients have a CAG triplet repeat expansion in the ATXN3 gene, we examined the effect of $(CUG)_7$ treatment in patient-derived SCA3 fibroblasts with a CAG CAA $(CAG)_n$ repeat where n = 18 and 72. As for htt, the $(CUG)_7$ treatment reduced the transcript from the expanded ataxin-3 allele, while reduction in transcript levels from the normal allele was less pronounced (**Figure 5a**).

PCR with primers amplifying a product containing the CAG repeat in ATXN3 showed a significant 97% (± 1%) down regulation of mutant ATXN3 after both 10nM and 100nM $(CUG)_7$ AON transfection (**Figure 5b**). The wild-type allele was reduced by respectively 27% (± 17%) and 33% (± 6%) by 10nM and 100nM after $(CUG)_7$ AON treatment.

Reduction on other expanded CAG transcripts by $(CUG)_7$ treatment

We next tested SCA1 and DRPLA fibroblasts. Allele-specific PCRs with primers flanking the CAG repeat were performed to examine the effect of $(CUG)_7$ treatment in both the normal and mutant allele. The mutant ataxin-1 (ATXN1) transcript was decreased by 89% (± 14%) in 100nM $(CUG)_7$ treated SCA1 cells compared to control transfections (**Figure 6a and c**), while the normal transcript was not reduced. (The SCA1 and DRPLA cell lines served as each other's control.) Mutant atrophin-1 (ATN1) in DRPLA was also reduced after 100nM $(CUG)_7$ treatment by 98% (± 2%), whereas there was only a 30% (± 6%) reduction in the normal allele (**Figure 6b and d**).

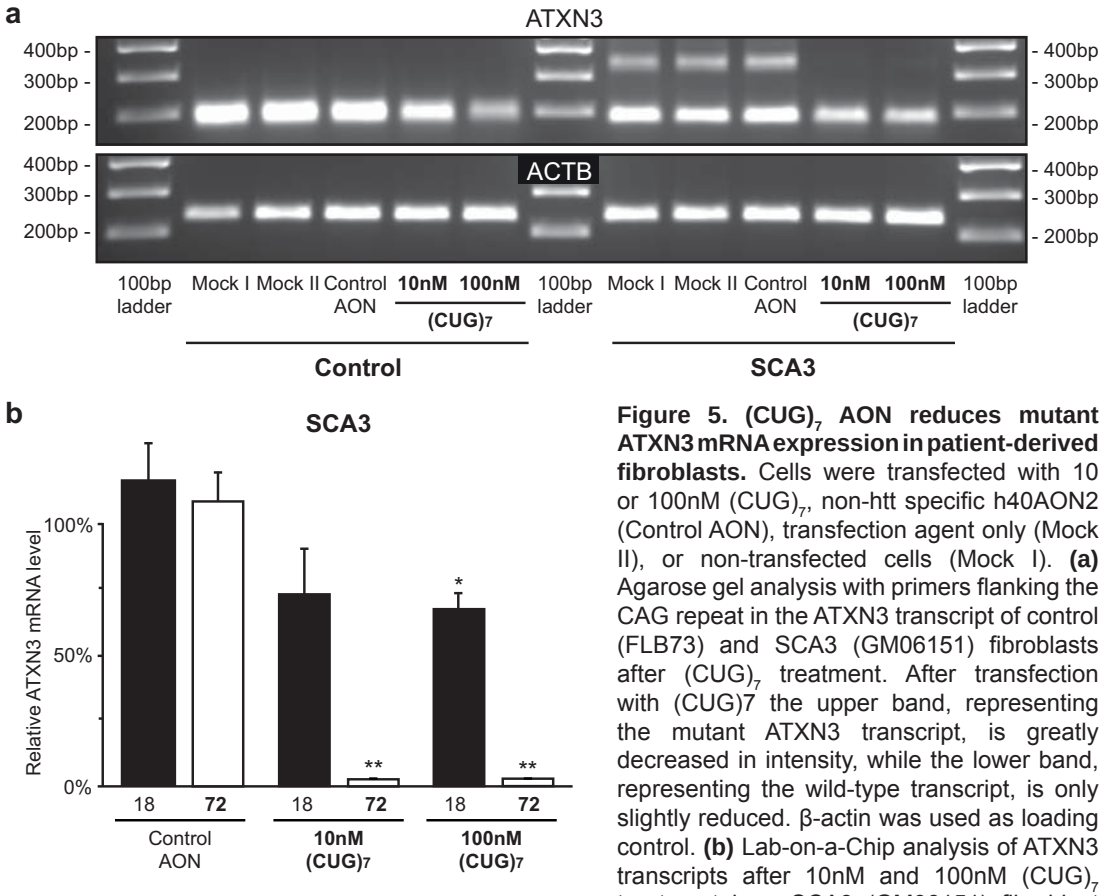

Figure 5. $(CUG)_7$ AON reduces mutant ATXN3 mRNA expression in patient-derived fibroblasts. Cells were transfected with 10 or 100nM $(CUG)_7$, non-htt specific h40AON2 (Control AON), transfection agent only (Mock II), or non-transfected cells (Mock I). **(a)** Agarose gel analysis with primers flanking the CAG repeat in the ATXN3 transcript of control (FLB73) and SCA3 (GM06151) fibroblasts after $(CUG)_7$ treatment. After transfection with (CUG)7 the upper band, representing the mutant ATXN3 transcript, is greatly decreased in intensity, while the lower band, representing the wild-type transcript, is only slightly reduced. β-actin was used as loading control. **(b)** Lab-on-a-Chip analysis of ATXN3 transcripts after 10nM and 100nM $(CUG)_7$ treatment in a SCA3 (GM06151) fibroblast cell line. The mutant transcript, with 72 CAGs, is significantly reduced by 97% after $(CUG)_7$ treatment, compared to transfection controls. The normal ATXN3 transcript with 18 CAGs is reduced by 27% and 33% after 10nM and 100nM $(CUG)_7$ AON treatment, respectively. Expression levels are corrected for loading differences with β-actin. The mRNA level of the Mock I transfection was set on 100% (* $P < 0.05$, ** $P < 0.01$, n = 2).

$(CUG)_7$ does not affect other endogenous CAG-enclosing transcripts

The human genome contains several proteins that contain polyQ tracts, usually encoded by a combination of CAG and CAA triplets. Most of these transcripts are essential for normal cellular function (Molla *et al.*, 2009) so reducing those transcripts could impair normal cellular function. To verify whether other uninterrupted CAG repeat containing transcripts were affected, 5 other transcripts were selected after a BLAST search: androgen receptor (AR), ataxin-2 (ATXN2), glutaminase (GLS), TBP, and zinc finger protein 384 (ZNF384). For the cells used in the present study the exact CAG tract length of these 5 transcripts was first determined by Sanger sequencing (**Table 1**). Primers for qPCR were designed within the CAG containing exon but amplifying a fragment downstream of the CAG repeat in the transcript (**Table S1**). For technical reasons primers for ATXN2 were designed upstream of the CAG repeat.

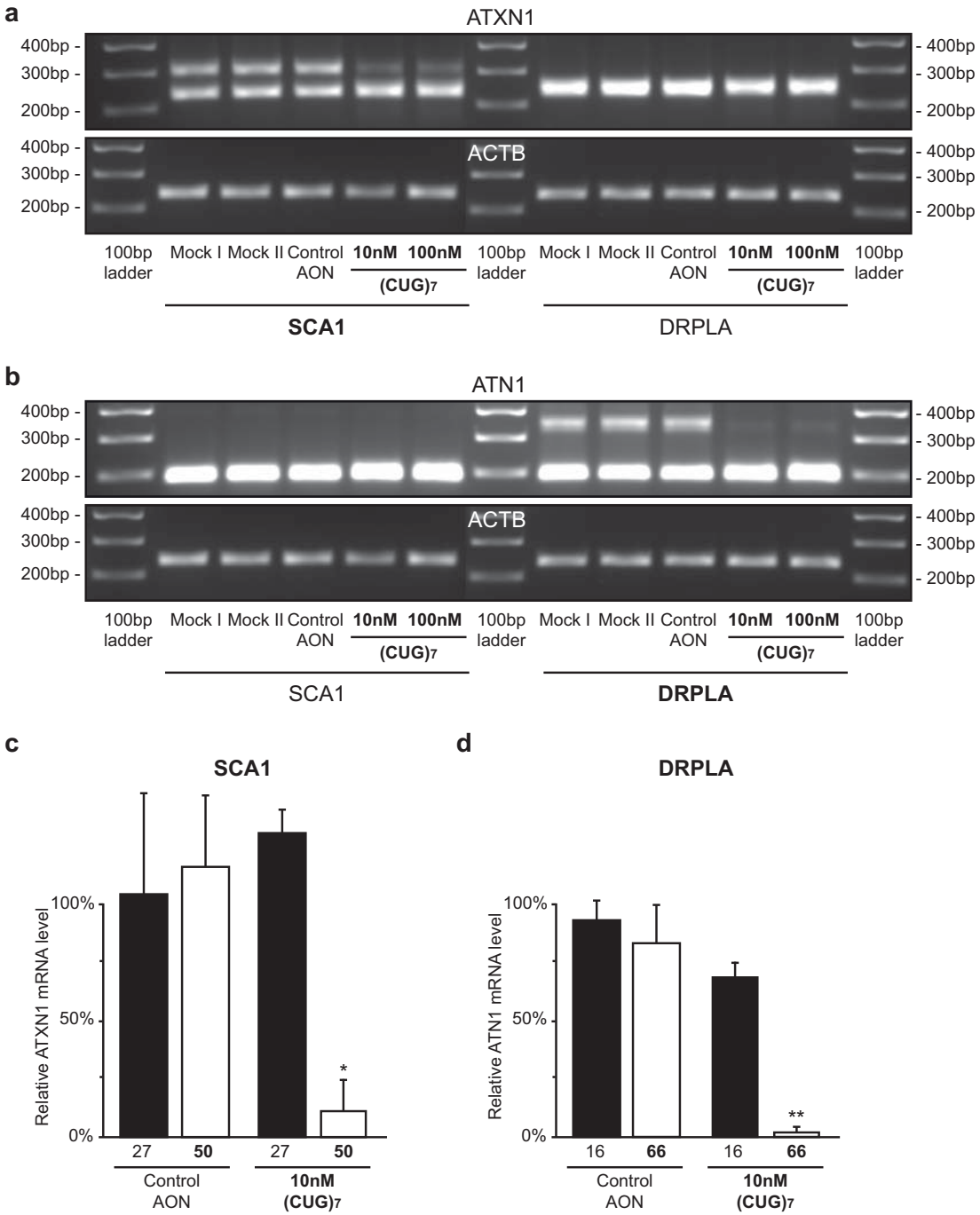

Figure 6. $(CUG)_7$ AON reduces mutant ATXN1 and ATN1 transcripts in SCA1 and DRPLA fibroblasts. SCA1 (GM06927) and DRPLA (GM13716) patient derived fibroblasts were transfected with 10 and 100nM $(CUG)_7$, 10nM non-htt specific h40 AON2 (Control AON), transfection agent only (Mock II), or non-transfected cells (Mock I). **(a)** Agarose gel analysis with primers flanking the CAG repeat in the ATXN1 transcript. After transfection with both 10nm and 100nM $(CUG)_7$ the upper band, representing the mutant ATXN1 transcript, is greatly decreased in intensity, while the lower band, representing the wild-type transcript, is not reduced. β-actin was used as loading control. **(b)** Agarose gel analysis with

primers flanking the CAG repeat in the ATN1 transcript. After transfection with both 10nM and 100nM $(CUG)_7$, the upper band representing the mutant ATN1 transcript, is greatly decreased in intensity, while the lower band representing the wild-type transcript, is not reduced. β-actin was used as loading control. **(c)** Lab-on-a-Chip analysis of ATXN1 transcripts in SCA1 cells after control AON and 10nM $(CUG)_7$ treatment. The mutant transcript, with 72 CAGs, is significantly reduced by 89% after $(CUG)_7$ treatment, compared to transfection controls. The normal ATXN1 transcript with 27 CAGs is not reduced. **(d)** ImageJ analysis of ATN1 transcripts in DRPLA cells after control AON and 10nM $(CUG)_7$ treatment. The 66 CAGs containing mutant ATN1 transcript is significantly reduced by 98% after $(CUG)_7$ treatment, while normal ATN1 transcript with 16 CAGs is not significantly reduced by 30%. Expression levels are corrected for loading differences with β-actin. The mRNA level of the Mock I transfection was set on 100% (* $P < 0.05$, ** $P < 0.01$, n = 3).

All tested CAG-enclosing transcripts were unaffected by 100nM $(CUG)_7$ treatment (**Figure 7**), including the AR transcript that contained CAG repeats of 21 and 23 CAGs. Endogenous ataxin-3 (with 17:18 Qs) and TBP (37:38 Qs) protein levels were unaffected by 100nM $(CUG)_7$ treatment (**Figure 8**). From the above results we can conclude that $(CUG)_7$ does not significantly reduce endogenous CAG containing transcripts and does not decrease endogenous polyQ-containing protein levels.

3.5. Discussion

The present study shows that an AON targeting CAG repeats and consisting of 7 CUGs significantly reduces protein and RNA levels of mutant htt in patient-derived fibroblast cell lines. This reduction was also seen, but to a lesser extends with $(CUG)_{12}$ but not with $(CUG)_3$. Although there was also a reduction of normal HTT transcript levels, the results show a preferential allele-specific reduction of mutant HTT in patient derived HD cells and this allele specificity was improved when AON concentration was lowered from 100nM to 10nM.

Furthermore, other non-expanded CAG-containing transcripts that were investigated were not affected by $(CUG)_7$ treatment. There was no reduction after $(CUG)_7$ treatment of the AR transcript that contained the longest tested uninterrupted CAG repeat, namely 21 and 23 CAGs. Normal HTT that contained 17 and 21 CAG repeats did show a reduction after $(CUG)_7$ treatment, suggesting that there are other factors besides the number of consecutive CAG triplets that determine $(CUG)_7$ efficacy.

The results with mutant ATXN1, ATXN3, and ATN1 confirmed the specificity of $(CUG)_7$ for transcripts with an expanded CAG tract in SCA1, 3, and DRPLA patient derived cells, respectively. Our results suggest that $(CUG)_7$ could

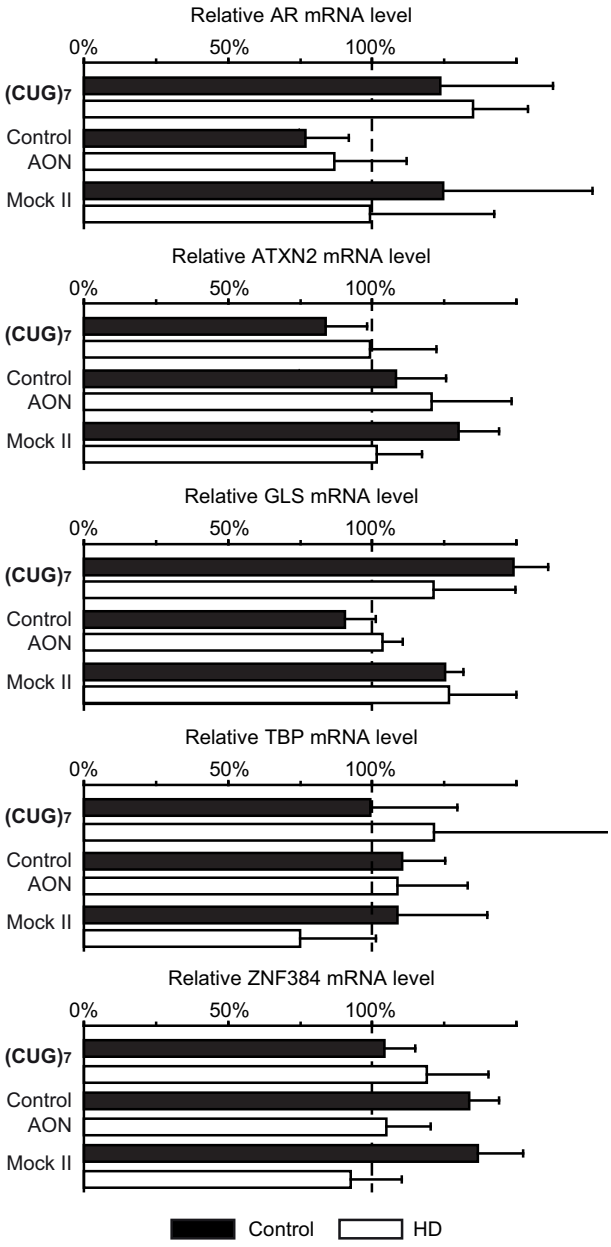

Figure 7. $(CUG)_7$ AON does not affect other CAG-containing transcripts. Quantitative real-time PCR was used to measure androgen receptor (AR), ataxin-2 (ATXN2), glutaminase (GLS), TATA box binding protein (TBP), and zinc finger protein 384 (ZNF384) mRNA levels in control and HD fibroblasts after treatment with 100nM $(CUG)_7$, non-htt specific h40AON2 (Control AON), transfection agent only (Mock II), or non-transfected cells (Mock I). All tested CAG-enclosing transcripts were unaffected by $(CUG)_7$ treatment. ACTB and RPL22 are used as reference genes. The expression level of Mock I transfections were set on 100% (n= 6)

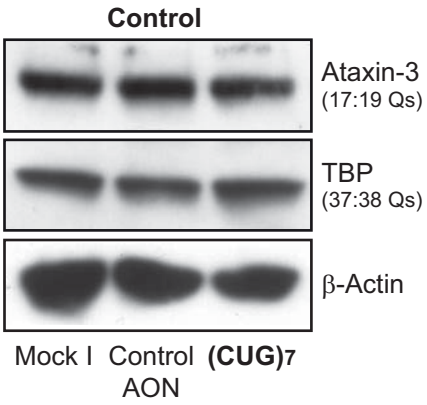

Figure 8. $(CUG)_7$ AON does not reduce other polyQ-containing proteins. Western blot of control (FLB73) fibroblasts treated with 100nM $(CUG)_7$, non-htt specific h40AON2 (Control AON), and non-transfected (Mock I). TATA box binding protein (TBP) and ataxin-3 are not reduced 72 hours after treatment with $(CUG)_7$. β-actin is used as loading control.

be effective in reducing expanded CAG repeat containing transcripts in all polyQ diseases.

In HD there is a gain of toxic function of the mutant htt protein, while regular htt expression is important for normal cellular function. Knockout of the homologous *htt* mouse gene was found to be early embryonic lethal (Zeitlin *et al.*, 1995) and previous studies have shown that approximately 50% of htt protein level is required to maintain cell functionality (Dragatsis *et al.*, 2000; Rigamonti *et al.*, 2000; Cattaneo *et al.*, 2001; Cattaneo *et al.*, 2005). In addition, increased clearance of mutant htt protein by autophagy in a Drosophila model and blockage of mutant htt in a conditional knock-out mouse model of HD resulted in a reduction in aggregates and an ameliorated phenotype (Yamamoto *et al.*, 2000; Sarkar *et al.*, 2007). Reduction of mutant protein levels will therefore most likely result in amelioration of the toxic HD phenotype but total knockdown of htt protein expression would not be advantageous (Sah and Aronin, 2011).

For other polyQ disorders the role of wild-type polyQ proteins in adult brain is still poorly understood. In a SCA3 Drosophila model expressing normal and mutant human ataxin-3, loss of normal ataxin-3 contributed to neurodegeneration (Warrick *et al.*, 2005). In contrast, non-allele-specific reduction of endogenous ataxin-3 was not found to be detrimental in rodents (Schmitt *et al.*, 2007; Alves *et al.*, 2010). Ataxin-1 knockout mice resulted in cerebellar transcriptional changes resembling SCA1 pathology, suggesting a neuroprotective role of normal ataxin-1 (Crespo-Barreto *et al.*, 2010). In contrast, atrophin-1 knockout mice were viable and did not show a clear phenotype (Shen *et al.*, 2007), suggesting that non-allele-specific reduction of both alleles in DRPLA is not harmful. Future research is necessary to determine the significance of wild-type polyQ protein levels for normal cellular function and the importance of AON-mediated allele-specific transcript reduction.

Several papers have shown allele-specific silencing of mutant htt with SNP-specific siRNAs (van Bilsen *et al.*, 2008; Zhang *et al.*, 2009b). Indeed HD patients carry different SNPs, requiring the development of at least five different siRNAs, to target 75% of the European and United States HD population (Lombardi *et al.*, 2009; Pfister *et al.*, 2009). However, the advantage of the approach described in the current paper is that it requires only 1 AON to treat all HD patients and would be applicable in other polyQ diseases. Furthermore, siRNAs are double stranded oligonucleotides and these have been described to cause off-target effects by the sense strand, (Fedorov *et al.*, 2006) as well as striatal toxicity (Grimm *et al.*, 2006; McBride *et al.*, 2008). In addition, RNA interference is an endogenous process; addition of siRNAs might cause toxicity due to an overload of the endogenous system. Recently, nucleic acids conjugates, with

Table 1. Number of uninterrupted CAGs and codons that encode for glutamine in CAG repeat enclosing transcripts as determined by Sanger sequencing and a summary of the effect of $(CUG)_7$ treatment in those transcripts.

Transcript Name	Cell Line	Glutamine stretch		Uninterrupted CAGs		Significant reduction after 100nM $(CUG)_7$ AON
		Allele 1	Allele 2	Allele 1	Allele 2	
AR	Control	22	24	21	23	No
	HD	23	24	22	23	No
ATN1	Control	19	20	15	16	No
	HD	12	19	8	15	No
	DRPLA	**20**	**70**	**16**	**66**	**Yes**
	SCA1	20	20	16	16	No
ATXN1	DRPLA	29	31	14	15	No
	SCA1	**29**	**52**	**14**	**37**	**Yes**
ATXN2	Control	20	20	8	8	No
	HD	20	20	8	8	No
ATXN3	**Control**	**17**	**19**	**15**	**17**	**Yes**
	HD	19	23	17	21	No
	SCA3	**20**	**74**	**18**	**72**	**Yes**
GLS	Control	8	14	8	14	No
	HD	7	18	7	18	No
HTT	**Control**	**19**	**23**	**17**	**21**	**Yes**
	HD	**20**	**46**	**18**	**44**	**Yes**
TBP	Control	37	38	17	18	No
	HD	35	36	16	17	No
ZNF384	Control	15	16	14	15	No
	HD	15	16	14	15	No

Abbreviations: AR, androgen receptor; ATN1, atrophin-1; ATXN1, ataxin-1; ATXN2, ataxin-2; ATXN3, ataxin-3; GLS, glutaminase; HTT, huntingtin; TBP, TATA box binding protein; ZNF384, zinc finger protein 384. Reduced transcripts after $(CUG)_7$ treatment are depicted in bold.

different chemistries than the AONs used in the current study, were used for allele-specific silencing of mutant htt. PNAs consisting of 1 guanine, followed by 6 CTGs, complementary to the CAG repeat, were found to specifically reduce mutant htt and ataxin-3 protein levels in patient-derived cells (Hu *et al.*, 2009a; Hu *et al.*, 2011). Although the reduction in protein levels by PNA transfection was highly efficient with very long stretches of CAGs, there was only a minor decrease when the number of CAG repeats that occur most frequently in the patient population was targeted (Hu *et al.*, 2009a; Hu *et al.*, 2009b). Testing a variety of modifications resulted in oligonucleotides with a thymine (T) LNA nucleotide at every third base (LNA(T)) and 2'O,4'O-C-ethyl nucleic acid (cET) which show higher selectivity (2.9 and 3.7 fold) for mutant alleles with 41 CAG repeats (Gagnon *et al.*, 2010).

AONs are a promising therapeutic tool, as was recently shown by phase I and phase I/II clinical trials in Duchenne muscular dystrophy (DMD) boys carrying specific deletions in the DMD gene (Goemans *et al.*, 2011). Local and systemic (subcutaneous) delivery of a specific 2OMe modified AON induced exon 51 skipping in the *DMD* gene on transcript level allowing the synthesis of novel, internally deleted, but likely (semi-) functional, dystrophin proteins without clinically apparent adverse events (van Deutekom *et al.*, 2007). AONs have also been used for the treatment of neurodegenerative disorders and are found to be taken up by neurons when delivered into the cerebral lateral ventricles. As treatment for ALS 2'-O-methoxyethyl modified deoxynucleotides infused intraventricularly were found to reduce both SOD1 transcript and protein levels in rats and rhesus monkeys, which resulted in a slower disease progression (Smith *et al.*, 2006). Similarly modified oligonucleotides for spinal muscular atrophy (SMA) resulted in putative therapeutic levels in all regions of the spinal cord after intrathecal infusion in non-human primates (Passini *et al.*, 2011).

The exact mechanism by which the AONs are used in the current study to reduce transcript levels and why they show both an allele and gene preference is not known. This selective repeat-length dependent reduction was also seen in myotonic dystrophy type 1 after $(CAG)_7$ AON treatment (Mulders *et al.*, 2009). Since 2OMe PS modified AONs are nuclease and RNase H resistant, RNase H-induced cleavage or RISC mediated degradation of dsRNA is not likely to be involved (Mulders *et al.*, 2009). Another explanation could be RNase H-independent translational blocking by $(CUG)_7$ AON binding to the transcript, preventing binding or steric blockage of the ribosomal units. However, translational blocking is not likely to be involved since htt transcript levels are also reduced (Hu *et al.*, 2009a). Reduction of transcript levels are not thought to be caused by interference of the $(CUG)_7$ AON during cDNA synthesis. Addition of $(CUG)_7$ AON just prior to the mRNA before cDNA synthesis did not result in reduced htt transcript levels (data not shown). A more likely explanation for the allele-specific effect of the $(CUG)_7$ AON shown in the current paper could be caused by structural differences in transcripts with normal and expanded repeats. Expanded CAG repeats are known to from hairpin structures (de Mezer *et al.*, 2011). $(CUG)_7$ AON binding could stabilize this CAG RNA hairpin, resulting in selective breakdown of the mutant transcripts. Another explanation could be that the expanded CAG repeats have a more open structure, making them more accessible for AON binding, thereby leading to induction of selective breakdown, resulting in a lower mRNA expression. These two models are not mutually exclusive and other mechanisms may as well be involved.

However, these results show that reduction of the mutant mRNA and/or its translation are promising generic routes towards therapy of triplet expansion diseases. Our future plans would be unraveling the exact mechanism of the reduction of HTT transcripts by the AON and *in vivo* testing of the toxicity and delivery of the $(CUG)_7$ in animal models of polyQ diseases.

Here we show the first evidence of a specific reduction of mutant huntingtin, ataxin-1 and -3, and atrophin-1 transcript levels using 2OMe PS modified AONs that recognizes pure CAG repeat stretches, suggesting that a single AON is potentially applicable to polyQ neurodegenerative diseases with an expanded pure CAG repeat.

3.6. Acknowledgments

Judith C.T. van Deutekom and Susan A.M. Mulders are employed by Prosensa Therapeutics B.V. (Leiden, The Netherlands). Prosensa Therapeutics B.V. holds a patent on the use of AONs to reduce CAG and GTG repeat containing transcripts and may (ultimately) benefit from the application of the submitted information. The LUMC does not have any patents on the reported area and has no financial relationship with Prosensa in the field of triplet expansion diseases, nor foresees to benefit otherwise from the use of the reported information.

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3.7. Supplementary Material

Table S1. Used primers for Sanger sequencing and (quantitative) RT-PCR.

Transcript	Direction	Amplifying the CAG repeat	Without CAG repeat
HTT	Forward	ATGGCGACCCTGGAAAAGCTGAT	ATGGCGACCCTGGAAAAG
	Reverse	TGAGGCAGCAGCGGCTG	CTGCTGCTGGAAGGACTTG
AR	Forward	GACCTACCGAGGAGCTTTCC	TGCAACTCCTTCAGCAACAG
	Reverse	CTCATCCAGGACCAGGTAGC	TCGAAGTGCCCCCTAAGTAA
ATXN1	Forward	TGGAGGCCTATTCCACTCTG	
	Reverse	TGGACGTACTGGTTCTGCTG	
ATXN2	Forward	CCTCACCATGTCGCTGAAG	CTCCGCCTCAGACTGTTTTG
	Reverse	GGAGACCGAGGACGAGGAC	GAGAAGGAGGACGACGAAGG
ATXN3	Forward	GAGCTTCGGAAGAGACGAGA	GGGGACCTATCAGGACAGAG
	Reverse	GATCACTCCCAAGTGCTCCT	CAAGTGCTCCTGAACTGGTG
ATN1	Forward	CACCCACCAGTCTCAACACA	TCACAGCCAGGTGTCCTACA
	Reverse	GAGACATGGCGTAAGGGTGT	GTAGCCGAAGAGGTGGTGAC
GLS	Forward	TAGGCGGAGCGAAGAGAAC	ACCCAAGTAGCTGCCCTTTC
	Reverse	GCTCAACAGGGGAGGATG	GCTCAACAGGGGAGGATG
TBP	Forward	GACCCCACAGCCTATTCAGA	CCACAGCTCTTCCACTCACA
	Reverse	TTGACTGCTGAACGGCTGCA	GCGGTACAATCCCAGAACTC
ZNF384	Forward	ACATATGCGCAAACACAACC	CCACCACACTTCCAGTCTCC
	Reverse	CCAGGAGACTGGAAGTGTGG	TGACAGTGAGGCAGATGTCC
ACTB	Forward		GGACTTCGAGCAAGAGATGG
	Reverse		AGCACTGTGTTGGCGTACAG
RPL22	Forward		TCGCTCACCTCCCTTTCTAA
	Reverse		TCACGGTGATCTTGCTCTTG

All primer sequences are from 5' - 3'. Abbreviations are as follows: AR, androgen receptor; ATN1, atrophin-1; ATXN1, ataxin-1; ATXN2, ataxin-2; ATXN3, ataxin-3; GLS, glutaminase; HTT, huntingtin; TBP, TATA box binding protein; ZNF384, zinc finger protein 384; ACTB, β-actin; RPL22: ribosomal protein L22.