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S. Metzger, C. Walter, Olaf Riess, Raymund a C Roos, J. Nielsen, D. Craufurd, Huu Nguyen, . Registry Investigators of The European Huntington'S Disease Network, Olivier Godefroy, Martine Roussel, et al.

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The V471A Polymorphism in Autophagy-Related Gene *ATG7* Modifies Age at Onset Specifically in Italian Huntington Disease Patients

Silke Metzger^{1,2}, Carolin Walter^{1,2}, Olaf Riess^{1,2}, Raymund A. C. Roos³, Jørgen E. Nielsen^{4,5}, David Craufurd⁶, REGISTRY Investigators of the European Huntington's Disease Network¹, Huu Phuc Nguyen^{1,2*}

1 Institute of Medical Genetics and Applied Genomics, University of Tuebingen, Tuebingen, Germany, **2** Rare Disease Center, University of Tuebingen, Tuebingen, Germany, **3** Department of Neurology, Leiden University Medical Centre, Leiden, The Netherlands, **4** Memory Disorders Research Unit, Neurogenetics Clinic, Section 6702, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark, **5** Institute of Cellular and Molecular Medicine, Section of Neurogenetics, University of Copenhagen, The Panum Institute, Copenhagen, Denmark, **6** Genetic Medicine, University of Manchester, Manchester Academic Health Sciences Centre and Central Manchester University Hospitals NHS Foundation Trust, St. Mary's Hospital, Manchester, United Kingdom

Abstract

The cause of Huntington disease (HD) is a polyglutamine repeat expansion of more than 36 units in the huntingtin protein, which is inversely correlated with the age at onset of the disease. However, additional genetic factors are believed to modify the course and the age at onset of HD. Recently, we identified the V471A polymorphism in the autophagy-related gene *ATG7*, a key component of the autophagy pathway that plays an important role in HD pathogenesis, to be associated with the age at onset in a large group of European Huntington disease patients. To confirm this association in a second independent patient cohort, we analysed the *ATG7* V471A polymorphism in additional 1,464 European HD patients of the "REGISTRY" cohort from the European Huntington Disease Network (EHDN). In the entire REGISTRY cohort we could not confirm a modifying effect of the *ATG7* V471A polymorphism. However, analysing a modifying effect of *ATG7* in these REGISTRY patients and in patients of our previous HD cohort according to their ethnic origin, we identified a significant effect of the *ATG7* V471A polymorphism on the HD age at onset only in the Italian population (327 patients). In these Italian patients, the polymorphism is associated with a 6-years earlier disease onset and thus seems to have an aggravating effect. We could specify the role of *ATG7* as a genetic modifier for HD particularly in the Italian population. This result affirms the modifying influence of the autophagic pathway on the course of HD, but also suggests population-specific modifying mechanisms in HD pathogenesis.

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* E-mail: hoa.nguyen@med.uni-tuebingen.de

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Introduction

Huntington disease (HD) is one of the most common monogenetic neurodegenerative disorders and is clinically characterized by progressive development of motor disturbances as well as cognitive and psychiatric dysfunctions mainly starting at middle age [1]. The underlying genetic defect of HD is the expansion of an unstable CAG repeat in the *HTT* gene resulting in an elongated polyglutamine tract of the huntingtin protein (htt), which is inversely correlated with the age-at-onset (AAO) and the course of the disease [2–4]. The length of the polyglutamine tract accounts for 42 to 73% of the variance in the AAO [5,6]. The remaining variance of AAO may be due to modifier genes and seems to be strongly heritable. However, environmental effects such as daily activity may also modify AAO in HD patients [7]. To date, several studies identified genetic modifiers of AAO in HD participating in glutamatergic transmission (*GRIK2*, *GRIN2A*,

GRIN2B) [8–11], axonal trafficking (*HAPI*) [12], gene transcription (*TCERG1*, *TP53*) [13,14], energy metabolism (*PPARGC1A*) [15–17] or protein degradation (*UCHL1*, *ATG7*) [18–20] representing various intracellular pathways involved in pathogenic processes of HD [21].

Recently, we found the V471A polymorphism in the *autophagy-related gene 7 (ATG7)* to be associated with the AAO of HD in a large group of more than 900 European HD patients [20]. The gene product of *ATG7* is an important part in the autophagic machinery facilitating the degradation of long-lived proteins, protein complexes and damaged organelles in the cell [22]. Additionally, autophagy enables the degradation of aggregate-prone proteins such as mutant huntingtin (mhtt), which tends to form intracellular aggregates and fails to undergo proteasomal degradation [23,24]. Our previous study revealed a significant effect of the *ATG7* V471A polymorphism on the HD AAO leading to an approximately 4-years-earlier onset of the first

symptoms [20]. The rare p.471A allele might affect ATG7 function and subsequently impairs the autophagic process and the degradation of mhtt. That ATG7 dysfunction could lead to neurodegeneration is supported by observations in ATG7-deficient mice, which exhibit a loss of cerebellar and cortical neurons and the formation of ubiquitin-positive aggregates [25,26]. On the other hand, a general induction of autophagy results both in a reduction of soluble and aggregated mhtt and protects against mhtt-mediated toxicity in cell, fly and mouse models of HD [23,27,28].

In order to validate a true association of a genetic modifier with the AAO of a disease or to facilitate a more detailed association analysis, independent replication studies are mandatory. Therefore, we analysed the association of the ATG7 V471A polymorphism with the HD AAO, which we detected in a previous study, in a second independent population composed of more than 1,400 European HD patients and specified the modifying effect of ATG7 in different populations.

Materials and Methods

HD Patients

As the aim of this study is the analysis of the identified modifier polymorphism ATG7 V471A in a second HD patients cohort, we examined a patients group consisting of 1,464 unrelated European HD patients, which were obtained by the European Huntington’s Disease Network (EHDN) “REGISTRY” study prior to February, 2011 (2nd European HD cohort = EHDN REGISTRY cohort). The EHDN REGISTRY project is a multicentre, prospective, observational study that enrolls HD expansion carriers [29]. For all patients HD was clinically diagnosed. Motor, psychiatric and cognitive signs were scored using the Unified Huntington’s Disease Rating Scale (UHDRS) and AAO was estimated as the onset of motor symptoms. The mean AAO was 43.8 (SD 12.3) and ranged from 5 to 78 years. CAG repeat lengths in the HD gene of the patients were mainly examined by a PCR amplification followed by capillary electrophoresis by BioRep (Milan, Italy). The number of the expanded CAG repeats ranged from 37 to 89 with a median repeat number of 43. The EHDN REGISTRY cohort includes patients from different European countries except Germany and Italy.

Furthermore, we analysed additional patients of a 1st European HD cohort that was already examined in a previous study [20]. This group consisted of a total of 943 European HD patients with subgroups of 371 patients of German and 327 patients of Italian descent. Compared to the number of investigated patients in the previous study [20] we managed it to re-genotype 25 HD patients of the 1st European HD cohort, which failed during the first genotyping. Also for all these patients, HD was clinically diagnosed and AAO was estimated as the age at which motor symptoms first occurred. The mean AAO of the 1st European cohort patients was 45.2 (SD 13.4) and ranged from 5 to 85 years. CAG repeat lengths of the expanded allele ranged from 39 to 90 units (median: 44). No overlap between the 1st European HD cohort and the EHDN REGISTRY cohort is expected.

Ethics Statement

The study was performed under a protocol approved by the Institutional Review Board of the University of Tuebingen Medical Faculty and the other sites of the EHDN REGISTRY project [29]. The participants gave informed written consent according to the International Conference on Harmonisation – Good Clinical Practice (ICH-GCP) guidelines (<http://www.ich.org/LOB/media/MEDIA482.pdf>) and according to the Declaration of Helsinki.

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Genotyping

As the polymorphism V471A in ATG7 was already analysed in a large HD patient cohort in a previous study [20], we used the same genotyping conditions as described. So genotyping of ATG7 V471A (dbSNP rs36117895) was performed by standard PCR conditions and a following restriction analysis using 1U MboII according to manufacturer’s instructions (New England Biolabs Inc., Beverly, MA, USA). The nomenclature for numbering of changes at nucleotide or amino acid level follows general rules [30].

Statistical Analysis

For a descriptive statistical analysis allele and genotype frequencies as well as Hardy-Weinberg distribution of the ATG7 V471A genotype was investigated by Genepop version 4.0.10 (<http://genepop.curtin.edu.au/>) (Table 1). Using the framework of linear models in an analysis of variance and covariance (JMP® Version 7.0.1, SAS Institute Inc., Cary, NC, USA), we tested the modifying role of the ATG7 V471A polymorphism on the AAO of HD. First, we applied a model of analysis of variance with the ATG7 V471A polymorphism and the expanded HD allele as independent variables and the AAO as dependent variable. The goodness of fit was evaluated by the proportion of variation in the AAO explained by the coefficient of determination (R²). We obtained the best fit of our data and a minimization of the residuals by logarithmic transformation of the AAO and the CAG repeat number in the HTT gene. To determine the effect of the ATG7 V471A polymorphism on AAO by an analysis of covariance in this model, the effect of the expanded CAG allele (HD CAG) was calculated alone, as well as with the V471A polymorphism. A change of R² after adding the effect of the polymorphism indicated a relative improvement of the model and thus identified the percentage of variance that was attributable to the ATG7 V471A polymorphism when there was a significant P value (P≤0.05). Adjustment of multiple testing was performed according to Bonferroni correction. Differences in the AAO of HD

Table 1. Allele and genotype frequencies of ATG7 V471A polymorphism.

Group	Allele frequency ^a		Genotype frequency		
	V	A	VV	VA	AA
EHDN REGISTRY cohort (n = 1464)	0.961	0.039	0.947	0.053	0.000
1 st European cohort (n = 918)	0.960	0.040	0.919	0.081	0.000
Controls (n = 60)	0.967	0.033	0.933	0.067	0.000

The nomenclature for numbering of changes at nucleotide or amino acid level follows general rules [30].

The observed genotypes did not differ from expectations under Hardy-Weinberg equilibrium (EHDN REGISTRY cohort: P = 1.000; 1st European cohort: P = 0.3920; controls: P = 1.000 [20]).

Genotype frequencies of Atg7 V471A in EHDN REGISTRY patients differ significantly from the respective frequencies in HD patients of the previous cohort (P = 0.0007).

n, number of investigated persons whose genotype could be determined.

^aAllele frequency of nucleotide substitution in ATG7 is described by V (= valine) as major allele and A (= alanine) as rare allele.

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within different genotypes were determined by a two-tailed *t* test (JMP® Version 7.0.1, SAS Institute Inc., Cary, NC, USA).

Results

Analysing the ATG7 V471A polymorphism in patients of the EHDN REGISTRY [28] cohort as a second cohort of European HD patients and comparing them with patients of a previously examined 1st European HD cohort [20] that does not overlap with the EHDN REGISTRY cohort, the respective alleles showed comparable frequencies. However, the heterozygous V471A genotype was significantly less frequent in the EHDN REGISTRY patients than in patients of the 1st European HD cohort (P=0.0007) (Table 1). The expanded CAG repeat in the *HTT* gene accounts for up to 66% of the variance in the AAO (R²=0.6633), so that about 34% of the AAO variance has to be determined by other factors acting as modifiers of the disease. In contrast to our previous study where we identified the ATG7 V471A polymorphism as a modifier of HD AAO, this polymorphism did not exert any overall significant influence on the AAO of the entire 1,464 EHDN REGISTRY patient cohort (Table 2). Similar results were obtained when analysing a potential effect of ATG7 V471A when grouping the patients according to longer and shorter CAG repeat lengths or sex (data not shown).

In order to check whether the difference on the influence of the ATG7 V471A polymorphism on HD AAO in the two independent cohorts could be attributed to the origin of the patients, i.e. the different European countries, we first analysed the impact of the patients' origin on their AAO. As this factor showed a highly significant effect on the HD AAO (P<0.0001), we split the HD patients of the EHDN REGISTRY cohort as well as the 1st European HD cohort by ancestry and analysed the impact of the ATG7 V471A polymorphism in these single groups. Remarkably, the population specific analysis of an ATG7 V471A effect on HD AAO revealed an influence of the polymorphism in addition to the expanded HD allele only in Italian HD patients (P=0.0119) (Table 3). In all other examined European populations ATG7 V471A did not exert any modifying effect on the disease onset. Also, the frequency of the heterozygous V471A genotype is significantly higher in Italian HD patients (P<0.05) than in the other populations, which could explain the higher heterozygosity rate in the 1st European HD cohort of our previous study (Table 1). The higher heterozygosity rate in Italian HD patients may have provided sufficient power to detect the effect of the ATG7 V471A polymorphism on the HD AAO in this population as other analysed populations, which are comparable in size (e.g. patients from the UK), have a significantly less amount of patients with a heterozygous V471A genotype.

Thereby, the heterozygous V471A genotype in Italian HD patients, who develop their first symptoms at an average age of 42.4 years, is associated with an approximately 6-years earlier

onset of the disease compared to patients homozygous for the major allele (Table 4).

Interestingly, Italian HD patients have the oldest mean AAO compared with patients from the other European countries analysed in this study, which differs significantly from the mean AAO of other European patients (Table 5). HD patients from Denmark and France show a comparatively similar AAO (P≥0.05), which is not modified by the genotype of the Atg7 V471A polymorphism.

In the Italian population, the expanded HTT allele itself accounts for ~46% (R²=0.4595) of the variance in the HD AAO, thus allowing a broader range for an additional influence of disease modifying factors. The ATG7 V471 polymorphism contributes ~1.7% to the variance in the AAO representing ~3% of the variance that cannot be accounted for by the expanded CAG repeat in the *HTT* gene. Interestingly, it can be observed that both in patients of the Italian population separately and in the whole EHDN REGISTRY cohort, the rare p.471A allele is, with the exception of one Italian patient, only present in patients with shorter HD alleles with ≤55 CAG units. Thus, the rare p.471A allele seems to assert its modifying effect in conditions with shorter and thus less severely affecting CAG expansions that potentially allow a greater influence of additional modifiers.

Discussion

The present study intended to confirm the modifying effect of the V471A polymorphism in the *ATG7* gene, previously identified in more than 900 European HD patients, in a second independent cohort of HD patients. Instead of a replication of our previous results in this second cohort of more than 1400 European HD patients, which is expected to have no overlap with the patients of our previous study, we could only detect the modifying effect of the ATG7 V471A polymorphism in Italian HD patients of our 1st European HD cohort. Here, the rare p.471A allele of the polymorphism is associated with a 6-years earlier onset of the first symptoms and thus potentially has an aggravating effect on the disease.

With this result, we had to revise and modify the finding of our previous study where we originally did not observe a population specific effect of the ATG7 V471A polymorphism [20]. Re-genotyping and addition of more patients to the analysis of the 1st European HD cohort lead to an exclusion of the ATG7 V471A influence on HD AAO in the German patients. This highlights the importance that the size of a collective needs to be as big as possible to indicate a real effect of a potential modifier.

In the analysed Italian population the modifying influence of the ATG7 V471A polymorphism seems to be robust. It represents about 3% of the variance in the AAO that cannot be accounted for by the expanded CAG repeat in the *HTT* gene itself. The CAG repeat determines about 46% of the whole variance in the HD AAO and thus is in accordance with a former study that analysed

Table 2. Analysis of covariance of ATG7 in EHDN REGISTRY cohort.

Model	R ²	ΔR ²	P value	Least significant number of patients ^a
CAGexp	0.6633		<0.0001	6
HD CAG+Atg7 V471A	0.6633	0.0000	0.6151	22244

The level of significance was set to P=0.05; n=1464.

CAGexp, expanded CAG allele in huntingtin.

^aMinimum number of patients, which are necessary to detect a significant effect of the analysed factor based on the respective genotypes.

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Table 3. Allele and genotype frequencies, Hardy-Weinberg distribution and analysis of covariance of ATG7 V471A in different European populations.

Country	Allele frequency ^a		Genotype frequency			Hardy-Weinberg	ANOVA ^c P-value
	V	A	VV	VA	AA		
Denmark (n = 101)	0.960	0.040	0.921	0.079	0.000	1.000	0.7797
France (n = 134)	0.981	0.019	0.963	0.037	0.000	1.000	0.8140
Netherlands (n = 174)	0.983	0.017	0.966	0.034	0.000	1.000	0.5537
Poland (n = 242)	0.988	0.012	0.975	0.025	0.000	1.000	0.3862
Spain (n = 168)	0.970	0.030	0.940	0.060	0.000	1.000	0.1481
UK (n = 347)	0.970	0.030	0.939	0.061	0.000	1.000	0.2611
Germany (n = 371) ^b	0.958	0.042	0.917	0.083	0.000	1.000	0.2988
Italy (n = 327)	0.946	0.054	0.893	0.107	0.000	0.6122	0.0238*

Populations with ≥100 examined HD patients are shown; n number of investigated HD patients.

^aAllele frequency of nucleotide substitution in ATG7 is described by V (= valine) as major allele and A (= alanine) as rare allele.

^bCompared to the number of investigated patients in the previous study [20] we managed it to re-genotype 25 HD patients of the 1st European HD cohort, which failed during the first genotyping.

^cAnalysis of covariance showing the effect of the ATG7 V471A polymorphism on the HD AAO in the respective population.

*significant effect, the level of significance was set to P=0.05 and the p-value was adjusted for multiple testing according to Bonferroni correction.

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the CAG repeat expansion in Italian HD families [31]. In comparison with different studies, the CAG repeat in the Italian population seems to have a relative weaker effect on the AAO [5,6,32] and additional factors may have a greater impact on the AAO. Depending on the population, the length of the CAG tract accounts for up to 73% of the variance in the AAO [5,6,32]. The remaining variance in the AAO, which is not accounted for by the CAG repeat, is determined by other genetic and environmental factors [33]. The impact of these additional factors also varies in different populations. Sibling analyses revealed a contribution of familial (genetic) factors to the HD AAO variance of up to 19% in addition to the expanded CAG repeat [34,35], but as a genetic factor modifies the HD AAO in one population, it can have less or even no influence in a second population. Examples of this are polymorphisms in the genes encoding the brain-derived neurotrophic factor (BDNF) [36–38], the glutamate receptor GRIK2 [8,9,39–41] or the CAG repeat length of the normal huntingtin allele [39,42–45]. Such a population-specific effect of disease modifying factors is also seen in other neurodegenerative diseases like Parkinson disease (PD) or Alzheimer disease. In this regard, BDNF shows a modifying effect in a cohort of Greek and Italian PD patients [46,47], but not in Finnish or Swedish patients [48,49].

The existence of population-specific effects of genetic modifiers in HD is supported by the identification of potential chromosomal modifier loci, which were achieved by genome wide linkage

analyses in different populations. The so called HD MAPS study, which analysed HD patients mainly from the United States and Canada, identified potential modifier loci on the chromosomal regions 4p16, 6p21–23, 6q23–26 and 18q22 [50,51]. A linkage scan in Venezuelan HD kindreds revealed loci at 2p25 and 2q35 as regions that harbour potential modifier genes, but could not confirm locus 18q22 of the HD MAPS study [52]. The chromosomal locus of ATG7 itself is on chromosome 3p25.3 and thus the gene is not located in any potential modifier region identified so far. It remains to be seen whether this region could be identified as a linkage locus when analysing HD patients particularly from Italy or Southern Europe.

Notably, a population specific effect of genetic modifiers on the HD AAO has been recently reported in the Italian population for the mitochondrial regulator PGC-1α [53]. We and others have observed that specific PPARGC1A SNPs are associated with the AAO of HD symptoms [15–17]. The modifying effect was mainly observable in Italian HD patients [16,17,53] and Ramos and coworkers suggested that this could be attributed to a population-dependent phenotype stratification as HD patients from Italy and Southern Europe were found to have a significantly older mean AAO than patients from other European regions [53]. Indeed, the Italian HD patients of the present study also showed a significantly older AAO than patients from most other European countries, although the sizes of the expanded CAG repeats are similar. While HD patients from France or Spain also show the tendency, though

Table 4. Mean ages-at-onset of the different Atg7 genotypes in Italian HD patients.

Genotype	Number of patients (n = 327)	Mean CAGexp (SD)	Mean AAO (SD)
VV	292	45.47 (4.73)	48.12 (13.87) ^a
VA	35	46.31 (7.66)	42.43 (14.68)
AA	–	–	–

CAGexp, expanded CAG repeat number in huntingtin, SD standard deviation, V major allele valine at amino acid position 471 (V471), A rare allele alanine at amino acid position 471 (A471).

^at test: patients with genotype VV differ significantly from patients with heterozygous VA genotype (P = 0.0348).

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Table 5. Mean ages at onset and CAG repeat numbers of the expanded huntingtin allele in different European populations.

Country	Mean AAO (SD)			T-test P-value ^a	Mean CAGexp (SD)		
	Atg7 genotype		all patients		Atg7 genotype		all patients
	VV	VA			VV	VA	
Denmark (n = 101)	46.53 (11.85)	46.13 (7.24)	46.50 (11.53)	n.s.	43.62 (4.70)	43.38 (2.07)	43.6 (4.51)
France (n = 134)	46.19 (12.09)	38.00 (11.81)	45.89 (12.14)	n.s.	44.18 (3.47)	47.00 (4.06)	44.28 (3.51)
Netherlands (n = 174)	44.42 (11.57)	46.00 (11.28)	44.45 (11.53)	n.s.	43.67 (3.12)	42.17 (1.17)	43.61 (3.08)
Poland (n = 242)	40.25 (13.93)	40.83 (15.54)	40.26 (13.93)	n.s.	46.11 (7.31)	44.50 (3.83)	46.07 (7.24)
Spain (n = 168)	43.04 (11.89)	51.10 (17.08)	43.52 (12.34)	n.s.	44.73 (3.98)	43.80 (5.03)	44.67 (4.04)
UK (n = 347)	44.93 (11.68)	42.19 (13.15)	44.76 (11.77)	n.s.	44.14 (3.69)	44.62 (3.57)	44.17 (3.68)
Germany (n = 371)	44.10 (12.77)	42.42 (14.74)	44.06 (12.90)	n.s.	45.61 (4.80)	45.90 (4.21)	45.61 (4.71)
Italy (n = 327)	48.12 (13.87)	42.43 (14.68)	47.52 (13.95) ^b	0.0348	45.47 (4.73)	46.31 (7.66)	45.56 (5.04)

Populations with ≥ 100 examined HD patients are shown; n number of investigated HD patients.

AAO, age at onset, CAGexp, expanded CAG repeat number in huntingtin, SD standard deviation, V major allele valine at amino acid position 471 (V471), A rare allele alanine at amino acid position 471 (A471).

^at test: Comparing the mean AAO of the VV and VA genotypes in the different populations, only Italian patients with genotype VV differ significantly from patients with heterozygous VA genotype ($P = 0.0348$).

^bt-test: Italian patients show the oldest mean AAO, which is significantly older than the mean AAO of patients from the other presented European countries together (Italy vs other countries: $P < 0.0001$), but not significantly different from the mean AAO of patients from Denmark ($P = 0.4728$) and France ($P = 0.2050$).

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not significant, for a marked difference in the AAO of ATG7 V471A genotypes compared to Italian HD patients their mean AAO is in the general AAO range of most other European populations. A later AAO, particularly in Southern Europe, may be due to further genetic factors, which modify the course of HD only in Southern European populations, but are not present in HD patients from other European regions. However, a later AAO may also reflect population differences, which could be due to genetic and/or environmental factors (such as method of diagnosis and lifestyle or nutrition) as were already discussed by Ramos and coworkers [53]. It is essential to consider such additional environmental factors in future investigations. However, examining the Italian patients of our study as a cohesive group, the method of diagnosing HD as well as other population specific environmental factors should be comparable. So, it may be possible that the ATG7 V471A polymorphism acts as a true genetic modifier in the Italian population. Most likely it seems to be in linkage disequilibrium with another genetic variation, potentially located in ATG7 itself. In this regard, the current study may also direct future modifier AAO studies in HD, such as more population stratification, stronger selection based on a more clearly defined AAO definition and even on selection of patients with a specific range of expanded polyQ length.

In conclusion, the findings presented here confirm a modifying role of the ATG7 V471A polymorphism on the AAO of HD, but with a specific effect in the Italian population. Despite this limitation, they provide further indication for a crucial link between autophagy and HD pathogenesis. However, further studies on the functional role of this potential genetic modifier are required to establish its role in HD pathogenesis and to potentially guide therapeutic approaches.

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The investigators of the European Huntington's Disease Network are:

Registry Steering committee

Anne-Catherine Bachoud-Lévi, **Université Paris Est, Faculté de Médecine, Créteil, France**; Anna Rita Bentivoglio, **Department of Neurology, Catholic University of Sacred Heart, Gemelli University Hospital, Rome, Italy**; Ida Biunno, **Institute of Genetics and Biomedical Research-National Research Council, Milan, Italy**; Raphael M. Bonelli, **Sigmund Freud University, Vienna, Austria**; Jean-Marc Burgunder, **Swiss Huntington's Disease Centre, Department of Neurology, University of Bern, Neurobu Clinics, Bern, Switzerland**; Stephen B. Dunnett, **Brain Repair Group, School of Biosciences, Cardiff University, Cardiff, UK**; Joaquim J. Ferreira, **Department of Neurology, Hospital de Santa Maria (CHLN, EPE), Lisbon, Portugal**; Olivia J. Handley, **The Brain Repair Group, School of Biosciences, Cardiff University, Cardiff, UK**; Arvid Heiberg, **University Hospital, Rikshospitalet, Department of Medical Genetics and Department of Neurology, Oslo, Norway**; T. Illmann, **Department of Computer Science, University of Rostock, Ulm, Germany**; G. Bernhard Landwehrmeyer, **Ulm University, Ulm, Germany**; John Levey, **Department of Medicine, Saint Vincent Hospital, University of Massachusetts Medical School, Worcester, USA**; Maria A. Ramos-Arroyo, **Complejo Hospitalario de Navarra, Pamplona, Spain**; Jørgen Nielsen, **University Hospital Copenhagen, Rigshospitalet, Memory Disorders Research Unit, Copenhagen, Denmark**; Susana Pro Koivisto, **Oslo University Hospital, Ullevål, Department of Medical Genetics, Oslo, Norway**; Markku Päivärinta, **Division of Clinical Neurosciences, Department of Neurology, University of Turku and Turku**

University Hospital, Turku, Finland; Raymund A.C. Roos, Leiden University Medical Centre (LUMC), Leiden, Netherlands; Ana Rojo Sebastián, University Hospital Mútua de Terrassa, Barcelona, Spain; Sarah Tabrizi, The National Hospital for Neurology and Neurosurgery, London, UK; Wim Vandenberghe, University Ziekenhuis Gasthuisberg Leuven, Belgium; Christine Verellen-Dumoulin, Institut de Pathologie et de Génétique, Charleroi, Belgium; Jacek Zaremba, Institute of Psychiatry and Neurology Department of Genetics, First Department of Neurology, Warsaw, Poland; Tereza Uhrová, Department of Psychiatry, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic; Jan Wahlström, Sahlgrenska University Hospital, Göteborg, Sweden

Language coordinators

Katrin Barth, **Innere Medizin 2, Klinikum Traunstein, Traunstein, Germany**; Leonor Correia-Guedes, **Hospital de Santa Maria, Neurological Clinical Research Unit, Institute of Molecular Medicine, Lisbon, Portugal**; Ana Maria Finisterra; Monica Bascuñana Garde, **Hospital Ramón y Cajal, Madrid, Spain**; Reineke Bos, **Leiden University Medical Centre (LUMC), Leiden, Netherlands**; Sabrina Betz, **Germany**; Jenny Callaghan, **Cardiff University, Schools of Medicine and Biosciences, Cardiff, UK**; Ruth Fullam, **Cardiff University, Schools of Medicine and Biosciences, Cardiff, UK**; Daniel Ecker, **Ulm University, Ulm, Germany**; Mette Gilling Nielsen, **Denmark**; Olivia J Handley, **Cardiff University, Schools of Medicine and Biosciences, Cardiff, UK**; Carina Hvalstedt; Christine Held, **University of Ulm, Ulm, Germany**; Kerstin Koppers, **France**; Matilde Laurà; Saul Martinez Horta, **Spain**; Asunción Martínez Descals, **Madrid-Fundación Jiménez Díaz, Madrid, Spain**; Tiago Mestre, **Hospital de Santa Maria, Neurological Clinical Research Unit, Institute of Molecular Medicine, Lisbon, Portugal**; Sara Minster, **Cardiff University, Schools of Medicine and Biosciences, Cardiff, UK**; Daniela Monza, **Foundation of Carlo Besta Neurological Institute, Milano, Italy**; Lisanne Mütze, **University of Ulm, Ulm, Germany**; Martin Oehmen, **University Hospital Muenster, Muenster, Germany**; Jenny Townhill, **Cardiff University, Schools of Medicine and Biosciences, Cardiff, UK**; Michael Orth, **Universitätsklinikum Hamburg-Eppendorf, Department of Neurology, Hamburg, Germany**; Helene Padiou, **France**; Laurent Paterski, **France**; Nadia Peppà, **The Royal Hallamshire Hospital-Sheffield Children's Hospital, Sheffield, UK**; Susana Pro Koivisto, **Oslo University Hospital, Ullevål, Department of Medical Genetics, Oslo, Norway**; Verena Roedig; Amandine Rialland, **Assistance Publique/Hôpitaux de Paris, Service de Neurologie et Faculté de Médecine Paris 12, Centre Hospitalier Universitaire Henri-Mondor, Créteil, France**; Niimi Røren, **Oslo University Hospital, Rikshospitalet, Norway**; Pavla Šašinková, **Czech Republic**; Yury Seliverstov, **Russia**; Patricia Trigo Cubillo, **Hospital Ramón y Cajal, Neurología, Madrid, Spain**; Marleen R van Walssem, **Oslo University Hospital, Rikshospitalet, Department of Medical Genetics and Department of Neurology, Oslo, Norway**; Abigail Wright; Wildson Vieira da Silva; Marie-Noelle Wijtes-Ané, **Leiden University Medical Centre, Leiden**; Bronovo hospital, **The Hague, The Netherlands**; Elizaveta Yudina, **V.G. Belinsky Penza State Pedagogic University, Penza, Russia**; Daniel Zielonka, **Poznan University of Medical Sciences, Poznan, Poland**; Eugeniusz Zielonka, **Balneological Department, Collegium Medicum, Jagiellonian University, Cracow, Poland**; Paola Zinzi, **Istituto di Neurobiologia e Medicina Molecolare & Istituto di Scienze e Tecnologie della Cognizione/CNR, Istituto di Neurologia Università Cattolica del Sacro Cuore, Rome, Italy**

Participating Investigators:

Raphael M. Bonelli, Brigitte Herranhof, Anna Holl, Hans-Peter Kapfhammer, Michael Koppitz, Markus Magnet, Daniela Otti, Annamaria Painold, Karin Reisinger, Monika Scheibl, Karen Hecht, Sabine Lilek, Nicole Müller, Helmut Schöggel, Jasmin Ullah, **University Hospital Graz, Graz, Austria**; Pascale Ribai, Christine Verellen-Dumoulin, **Institut de Pathologie et de Génétique, Charleroi, Belgium**; Andrea Boogaerts, Wim Vandenberghe, Dimphna van Reijen, **University Ziekenhuis Gasthuisberg, Leuven, Belgium**; Jiří Klempíř, Veronika Majerová, Jan Roth, **Department of Neurology 1st Faculty of Medicine and Teaching Hospital, Prague, Czech Republic**; Jørgen Nielsen, Lena Hjermland, Oda Jacobsen, Tua Vinthev-Jensen, Ida Unmack Larsen, Jette Stockholm, **University Hospital**

Copenhagen, Rigshospitalet, Memory Disorders Research Unit, Copenhagen, Denmark; Heli Hiivola, Kirsti Martikainen, Katri Tuuha, **Rehabilitation Centre Suviutuuli, Turku-Suviutuuli, Finland**; Jaakko Ignatius, Mikko Kärppä, Jaana Åman, **University of Oulu, Department of Neurology, Oulu, Finland**; Aki Mustonen, Outi Kajula, **University of Oulu, Department of Medical Genetics, Oulu, Finland**; Maire Santala, **Terveystalo Healthcare Service Centre, Tampere, Finland**; Philippe Allain, Marie-Anne Guérid, Bénédicte Gohier, Audrey Olivier, Adriana Prundean, Clarisse Scherer-Gagou, Christophe Verny, Marie Bost, **Centre Hospitalier Universitaire d'Angers, Centre de référence des maladies neurogénétiq, Angers, France**; Blandine Babiloni, Sabrina Deb-ruxelles, Charlotte Duché, Cyril Goizet, Danielle Lafoucrière, Laetitia Jameau, Umberto Spampinato, **Hôpital Pellegrin, Bordeaux, France**; Christelle De Bruycker, Maryline Cabaret, Anne-Sophie Carette, Luc Defebvre, Eric Decorte, Arnaud Delval, Marie Delliaux, Alain Destee, Kathy Dujardin, Mireille Peter, Lucie Plomhouse, Bernard Sablonnière, Clémence Simonin, Luc Defebvre, Marie-Hélène Lemaire, Sylvie Manouvrier, Stéphanie Thibault-Tanchou, Isabelle Vuillaume, **Hôpital Roger Salengro, Lille, France**; Pierre Krystkowiak, Cécile Duru, Martine Roussel, Sandrine Wannepain, Hassan Berrissoul, Marcellin Bellonet, Françoise Courtin, Béatrice Mantaux, Véronique Fasquel, Olivier Godefroy, **CHU Nord, Amiens, France**; Jean-Philippe Azulay, Frédérique Fluchère, Marie Delfini, Alexandre Eusebio, Laura Mundler, **Hôpital La Timone, Marseille, France**; Nadine Longato, Gabrielle Rudolf, Gisèle Steinmetz, Christine Tranchant, Caroline Wagner, Marie-Agathe Zimmermann, Christophe Marcel, **Hôpital Civil, Strasbourg, France**; Jürgen Andrich, Gisa Ellrichmann, Rainer Hoffmann, Barbara Kaminski, Carsten Saft, Christiane Stamm, **Huntington Centre (NRW), St. Josef-Hospital, Bochum, Germany**; Kai Boelmans, Christos Ganos, Ines Goerendt, Ute Hidding, Jan Lewerenz, Alexander Münchau, Michael Orth, Jenny Schmalfeld, Lars Stubbe, Simone Zittel, **Universitätsklinikum Hamburg-Eppendorf, Department of Neurology, Hamburg, Germany**; Kathrin Bürk, Jens Carsten Möller, Ida Rissling, **University of Marburg, Department of Neurology, Marburg, Germany**; Claudia Cormio, Vittorio Scruicchio, Claudia Serpino, Marina de Tommaso, **University of Bari, Neurophysiopathology of Pain Unit, Bari, Italy**; Sabina Capellari, Pietro Corelli, Roberto Gallassi, Roberto Poda, Giovanni Rizzo, Cesa Scaglione, University of Bologna, **Department of Neurological Sciences, Bologna, Italy**; Giovanni Abbruzzese, Monica Bandettini di Poggio, Emilio Di Maria, Giovanna Ferrandes, Paola Mandich, Roberta Marchese, **University of Genova; Department of Neuroscience, Oftalmologia e Genetica (DiNOG), Genova, Italy**; Alberto Albanese, Daniela Di Bella, Stefano Di Donato, Cinzia Gellera, Silvia Genitrini, Caterina Mariotti, Daniela Monza, Lorenzo Nanetti, Dominga Paridi, Paola Soliveri, Chiara Tomasello, **Foundation of Carlo Besta Neurological Institute, Milano, Italy**; Ferdinando Squitieri, Francesca Elifani, Vittorio Maglione, Alba Di Pardo, Silvia Alberti, Annamaria Griguoli, Enrico Amico, Tiziana Martino, **Centro di Neurogenetica e Malattie Rare IRCCS Neuromed, Pozzilli (IS), Italy**; Martina Petrolini, **Istituto Leonarda Vaccari, Rome, Italy**; Anna Rita Bentivoglio, Claudio Catalli, Raffaella Di Giacomo, Alfonso Fasano, Marina Frontali, Arianna Guidubaldi, Tamara Ialongo, Gioia Jacopini, Giovanna Loria, Carla Piano, Piccininni Chiara, Davide Quaranta, Silvia Romano, Francesco Soletti, Maria Spadaro, Paola Zinzi, **Istituto di Neurobiologia e Medicina Molecolare & Istituto di Scienze e Tecnologie della Cognizione/CNR, Istituto di Neurologia Università Cattolica del Sacro Cuore, Rome, Italy**; Monique S.E. van Hout, Jeroen P.P. van Vugt, A. Marit de Weert, **Medisch Spectrum Twente, Enschede, Netherlands**; J.J.W. Bolwijn, M. Dekker, K.L. Leenders, HPH Kremer, **Polikliniek Neurologie, Groningen, Netherlands**; Reineke Bos, Eve M. Dumas, Simon J. A. van den Bogaard, Raymund A.C. Roos, Ellen P. 't Hart, Erik van Duijn, **Leiden University Medical Centre (LUMC), Leiden, Netherlands**; Berry Kremer, C.C.P. Verstappen, **Universitair Medisch Centrum St. Radboud, Neurology, Nijmegen, Netherlands**; Ellen Økland Blinkenberg, **Haukeland University Hospital, Bergen, Norway**; Erik Hauge, Hilde Tyvoll, **NKS Olavikens HD clinic, Bergen, Norway**; Arvid Heiberg, Marleen R van Walssem, Jan Frich, Olaf Aaserud, Raghild Wehus, **Oslo University Hospital, Rikshospitalet, Department of Medical Genetics and Department of Neurology, Oslo, Norway**; Kathrine Bjørge, Madeleine Fannemel, Per Gørvell,

Eirin Lorentzen, Susana Pro Koivisto, Lars Retterstøl, Torborg Overland, Bodil Stokke, **Oslo University Hospital, Ullevål, Department of Medical Genetics, Oslo, Norway**; Inga Bjørnevoll, Signrid Botne Sando, **St. Olavs Hospital, Trondheim, Norway**; Artur Dziadkiewicz, Malgorzata Nowak, Piotr Robowski, Emilia Sitek, Jaroslaw Slawek, Witold Soltan, Michal Szinwelski, **St. Adalbert Hospital, Medical University of Gdansk, Neurological and Psychiatric Nursing Department, Gdansk, Poland**; Magdalena Blaszczyk, Magdalena Boczarska-Jedynak, Ewelina Ciach-Wysocka, Agnieszka Gorzkowska, Barbara Jasinska-Myga, Gregorz Opala, Gabriela Klodowska-Duda, Daniel Stempel, **Medical University of Silesia, Katowice, Poland**; Krzysztof Banaszekiewicz, Dorota Boćwińska, Andrzej Szczudlik, Monika Rudzińska, Magdalena Wójcik, Malgorzata Dec, Malgorzata Krawczyk, Kamila Bojakowska-Jaremek, Elżbieta Szczygieł, Agata Stenwak, Anna Wasielewska, **Krakowska Akademia Neurologii, Krakow, Poland**; Anna Bryl, Anna Ciesielska, Aneta Klimberg, Jerzy Marcinkowski, Justyna Sempolowicz, Daniel Zielonka, Husam Samara, Bartłomiej Wiśniewski, **Poznan University of Medical Sciences, Poznan, Poland**; Piotr Janik, Anna Gogol, Hubert Kwiecinski, Zygmunt Jamrozik, Anna Kaminska, **Medical University of Warsaw, Neurology, Warsaw, Poland**; Jakub Antezak, Katarzyna Jachimska, Maryla Rakowicz, Przemyslaw Richter, Rafal Rola, Danuta Ryglewicz, Halina Sienkiewicz-Jarosz, Iwona Stepniak, Grzegorz Witkowski, Elżbieta Zdzienicka, Jacek Zaremba, Anna Sulek, Wioletta Krysa, Iwona Stepniak, Karolina Zieora-Jakutowicz, **Institute of Psychiatry and Neurology Department of Genetics, First Department of Neurology, Warsaw, Poland**; Filipa Júlio, Cristina Januário, **University Hospital of Coimbra, Coimbra, Portugal**; Tiago Mestre, Leonor Correia-Guedes, Miguel Coelho, Tiago Mendes, Anabela Valadas, Joaquim J Ferreira, **Hospital de Santa Maria, Neurological Clinical Research Unit, Institute of Molecular Medicine, Lisbon, Portugal**; Carlos Andrade, Miguel Gago, Carolina Garrett, Maria Rosália Guerra, Joana Lima, João Massano, Joana Meireles, **Centro Hospitalar de São João, Faculdade de Medicina da Universidade do Porto, Porto, Portugal**; Carmen Durán Herrera, Patrocinio Moreno Garcia, **Hospital Infanta Cristina, Badajoz, Spain**; Francisco Barrero, Blas Morales, **Hospital Universitario San Cecilio, Neurología, Granada, Spain**; Esther Cubo, Natividad Mariscal, Jesús Sánchez, **Servicio de Neurología Hospital General Yagüe, Burgos, Spain**; Fernando Alonso-Frech, María Rabasa Perez, **University Hospital of Fuenlabrada, Fuenlabrada, Spain**; María Fenollar, Rocío García-Ramos García, Purificación Pin Quiroga, Susana Vázquez Rivera, Clara Villanueva, **Hospital Clínico Universitario San Carlos, Madrid, Spain**; Javier Alegre, Mónica Bascuñana, Juan Garcia Caldentey, Marta Fatás Ventura, Guillermo García Ribas, Justo García de Yébenes, José Luis López-Sendón Moreno, Patricia Trigo Cubillo, **Hospital Ramón y Cajal, Neurología, Madrid, Spain**; Pedro J García Ruíz, Asunción Martínez-Descals, María José Saiz Artiga, Vicenta Sánchez, Rosa Guerrero, Antonio Herranz Bárcenas, **Madrid-Fundación Jiménez Díaz, Madrid, Spain**; María Fuensanta Noguera Perea, Lorenza Fortuna, María Martirio Antequera Torres, Gema Reinante, Laura Vivancos Moreau, **Hospital Universitario Virgen de la Arrixaca, Murcia, Spain**; Miquel Aguilar Barbera, Dolores Badenes Guia, Laura Casas Hernanz, Judit López Catena, Ana Rojo Sebastián, Pilar Quilés Ferrer, Gemma Tome Carruesco, **University Hospital Mútua de Terrassa, Barcelona, Spain**; Jordi Bas, Núria Busquets, Matilde Calopa, **Bellvitge University Hospital, Barcelona, Spain**; Marina Dalmau Elorza, Cristóbal Díez-Aja López, Santiago Durán-Sindreu Terol, Misericordia Floriach Robert, Belén Garzón Ruíz, Ana González Casado, Isabel Haro Martínez, Celia Mareca Viladrich, Regina Pons i Cárdenas, Elvira Roca, Joan Roig Llesoy, Jesús Miguel Ruiz Idiago, Mar Ruiz Vergara, Socorro Soriano García, Antonio Villa Riballo, **Hospital Mare de Deu de La Merced, Barcelona, Spain**; Sonia González González, Luis Menéndez Guisasaola, Carlos Salvador, Esther Suárez San Martín, **Hospital Universitario Central de Asturias, Oviedo, Spain**; Mónica González, Aranzazú Gorospe, Inés Legarda, Penelope Navas Arques, María José Torres Rodríguez, Barbara Vives, **Hospital Son Dureta, Palma, Spain**; Itziar Gaston, Maria A. Ramos-Arroyo, Maria Dolores Martínez-Jaurrieta, **Complejo Hospitalario de Navarra, Pamplona, Spain**; Jose Manuel Garcia Moreno, José Chacón Peña, Luminita Dinca Avarvareî, Antonio Martín Bastida, María Fernández Recio, Luis Redondo Vergé, Violeta Sánchez Sánchez, **Hospital Universitario Virgen Macarena, Sevilla, Spain**; Fátima Carrillo, María Teresa

Cáceres, Pablo Mir, María José Lama Suarez, **Hospital Universitario Virgen del Rocío, Sevilla, Spain**; Ghada Loutfi, Carina Olofsson, Eva-Lena Stattin, Laila Westman, Birgitta Wikström, **Umeå University Hospital, Umeå, Sweden**; Sven E Pålhagen, Martin Paucar, Per Svenningsson, Tina Walldén Reza-Soltani, Arja Höglund, Britta Sandström, **Karolinska University Hospital, Stockholm, Sweden**; Jan Wahlström, Ulrika Høsterey-Ugander, Gunnel Fredlund, Radu Constantinescu, Liselotte Neleborn-Lingefjård, **Sahlgrenska University Hospital, Göteborg, Sweden**; Jean-Marc Burgunder, Yanik Stebler, **Swiss HD Zentrum, Bern, Switzerland**; Alain Kaelin, Irene Romero, Michael Schüpbach, Sabine Weber Zaugg, **University of Bern, Zentrum für Bewegungsstörungen, Neurologische Klinik und Poliklinik, Bern, Switzerland**; Zosia Miedzybrodzka, Daniela Rae, Lorna Downie, Sheila Simpson, Fiona Summers, Alexandra Ure, Roisin Jack, Kirsty Matheson, **NHS Grampian Clinical Genetics Centre & University of Aberdeen, Aberdeen, UK**; Shahbana Akhtar, Jenny Crooks, Adrienne Curtis, Jenny de Souza (Keylock), Hugh Rickards, Jan Wright, **The Barberry Centre, Department of Psychiatry, Birmingham, UK**; Roger A. Barker, Deidre O'Keefe, Anna Di Pietro, Kate Fisher, Anna Goodman, Susan Hill, Sarah Mason, Rachel Swain, Natalie Valle Guzman, **Cambridge Centre for Brain Repair, Forvie Site, Cambridge, UK**; Jonathan Bisson, Monica Busse, Cynthia Butcher, Jenny Callaghan, Catherine Clenaghan, Stephen Dunnett, Ruth Fullam, Olivia Handley, Sarah Hunt, Alis Hughes, Catherine Johnstone, Lesley Jones, Una Jones, Hanan Khalil, Sara Minster, Michael Owen, Kathleen Price, Linda Ellison Rose, Jenny Townhill, Anne Rosser, **Cardiff University, Schools of Medicine and Biosciences, Cardiff, UK**; Mary Porteous, Maureen Edwards, Carrie Ho, Marie McGill, Pauline Pearson, **Western General Hospital, SE Scotland Genetic Service, Edinburgh, UK**; Peter Brockie, Jillian Foster, Nicola Johns, Sue McKenzie, Jean Rothery, Gareth Thomas, Shona Yates, **Scottish Huntington's Association Whyteman's Brae Hospital, Fife, UK**; Liz Burrows, Amy Fletcher, Alison Harding, Fiona Laver, Mark Silva, Aileen Thomson, **Gloucestershire Royal Hospital, Department of Neurology, Gloucester, UK**; Liz Rowett, Deena Gallantrae, Mandy Longthorpe, Ivana Markova, Ashok Raman, Stephanie Hamer, Sue Wild, Pam Yarduiman, Carol Chu, Alison Kraus, **Castle Hill Hospital, Hull, UK**; Sue Wild, Pam Yardumian, Hannah Musgrave, Liz Rowett, Jean Toscano, Stuart Jamieson, Emma Hobson, **Chapel Allerton Hospital, Department of Clinical Genetics, Leeds, UK**; Carole Clayton, Heather Dipple, Julia Middleton, Dawn Freire-Patino, **Leicestershire Partnership NHS Trust, Leicester, UK**; Thomasin Andrews, Andrew Dougherty, Fred Kavalier, Charlotte Golding, Hana Laing, Alison Lashwood, Dene Robertson, Deborah Ruddy, Anna Whaite, Alastair Santhouse, **Guy's Hospital, London, UK**; Michael Patton, Maria Peterson, Sarah Rose, **St. Georges-Hospital, London, UK**; Thomasin Andrews, Stefania Bruno, Elvina Chu, Karen Doherty, Charlotte Golding, Salman Haider, Davina Hensman, Nayana Lahiri, Monica Lewis, Marianne Novak, Aakta Patel, Nicola Robertson, Elisabeth Rosser, Sarah Tabrizi, Rachel Taylor, Thomas Warner, Edward Wild, **The National Hospital for Neurology and Neurosurgery, London, UK**; David Craufurd, Ruth Fullam, Liz Howard, Andrea Sollom, Julie Snowden, Jennifer Thompson, Jenny Callaghan, Mary Jones, Helen Murphy, Iris Trender-Gerhard, Dawn Rogers, Judith Bek, Emma Oughton, Liz Johnson, Marianne Hare, Natalie Arran, Nichola Verstraeten, Lucy Partington-Jones, Susan Huson, Cheryl Stopford, Leann Westmoreland, **University of Manchester, Manchester Academic Health Sciences Centre and Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK**; Jill Davidson, Karen Morgan, Lois Savage, Baldev Singh, Suresh Komati, **Newcastle upon Tyne Hospitals, Newcastle, UK**; Andrea H Nemeth, Richard Armstrong, Ruth Valentine, Gill Siuda, **Oxford University Hospitals NHS Trust, Department of Neurosciences, Oxford, UK**; David Harrison, Max Hughes, Andrew Parkinson, Beverley Soltysiak, **Mount Gould Hospital, Plymouth Huntington Disease Service, Plymouth, UK**; Oliver Bandmann, Alyson Bradbury, Paul Gill, Helen Fairtlough, Kay Fillingham, Isabella Foustanos, Mbombe Kazoka, Kirsty O'Donovan, Nadia Peppia, Catherine Taylor, Katherine Tidswell, Oliver Quarrell, **The Royal Hallamshire Hospital-Sheffield Children's Hospital, Sheffield, UK**

Author Contributions

Conceived and designed the experiments: SM HPN. Performed the experiments: SM CW. Analyzed the data: SM. Contributed reagents/

materials/analysis tools: RACR JEN DC RIEHDN. Wrote the paper: SM. Reviewed the paper and provided comment: OR RACR JEN DC HPN.

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