Intermediate and expanded HTT alleles and risk for α-synucleinopathies

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Intermediate and expanded *HTT* alleles and the risk for α-synucleinopathies

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The authors report no conflicts of interest related to this work.

Abstract

Background: Previous studies suggest a link between CAG repeat number in the *HTT* gene and non-Huntington neurodegenerative diseases.

Objective: To analyze whether expanded HTT CAG alleles and/or their size are associated with the risk for developing α-synucleinopathies or their behavior as modulators of the phenotype.

Methods: We genotyped the *HTT* gene CAG repeat number and APOE-Ɛ isoforms in a case-control series including patients with either clinical or neuropathological diagnosis of α-synucleinopathy.

Results: We identified three PD patients (0.30%) and two healthy controls (0.19%) carrying low-penetrance *HTT* repeat expansions whereas none of the DLB or MSA patients carried pathogenic *HTT* expansions. In addition, a clear increase of the number of *HTT* CAG repeats was found among DLB and PD groups influenced by the male gender and also by the *APOE4* allele among DLB patients. *HTT* Intermediate alleles' (IAs) distribution frequency was increased in the MSA group compared with controls (8.8% vs 3.9%, respectively). These differences were indeed statistically significant in the MSA group with neuropathological confirmation. Two MSA *HTT* CAG IAs carriers with 32 *HTT* CAG repeats showed isolated polyQ inclusions in pons and basal nuclei, which are two critical structures in the neurodegeneration of MSA.

Conclusions: Our results pointed to a link between, *HTT* CAG number, *HTT* IAs and expanded *HTT* CAG repeats with other non -HD brain pathology and support the hypothesis that they can share common neurodegenerative pathways.

1-Introduction

The most frequent α-synucleinopathies, which are characterized by α-synuclein (α-syn) brain aggregates are Parkinson disease (PD), dementia with Lewy bodies (DLB) and multisystem atrophy (MSA). MSA is characterized by glial cytoplasmic α-synuclein (a-syn) inclusions, whereas in DLB and PD by intraneuronal Lewy bodies.[1] The brain exam from patients with α-synucleinopathies show very often concurrence of many other neurodegenerative diseases with tau or amyloid deposits.[2,3] Hence, it is possible that α-synucleinopathies also share other neuropathogenic mechanisms related to α-syn pathways that can be reflected in their genetic architecture.

Huntington disease (HD) is an inherited neurodegenerative disease linked to a CAG repeat expansion at the *Huntingtin* gene *(HTT*; MIM:613004). CAG *HTT* allele expansions above 36 can cause HD and above 41 are fully penetrant, whereas CAG *HTT* intermediate alleles (IAs; range 27-35) are unstable and are prone to increase their length into a pathological range in the offspring.[4] Recently, a putative etiologic role of the CAG repeats in the *HTT* gene has been proposed for non-Huntington neurodegenerative diseases as shown in an analysis of *HTT* gene CAG repeats in a large cohort of frontotemporal dementia/amyotrophic lateral sclerosis (FTD/ALS) and DLB, that showed fully-penetrant pathological *HTT* CAG gene expansions in 0.12-0.14% of FTD/ALS cohorts but not among DLB patients.[5] In addition, previous studies from our group found that IAs in *HTT* can have a role in AD risk[6] and CAG IAs in the *ATXN1, ATXN2* and *HTT* genes showed a variable frequency in different FTD subtypes.[7] These results further pointed towards a link between IAs and tauopathies, suggesting an involvement of *HTT* CAG expanded alleles in other neurodegenerative diseases.

To unravel the genetic architecture of α-synucleinopathies is critical for the design of clinical trials and will contribute to develop new drugs and disease modifying interventions. Thus, the main objective of this study is to determine whether the *HTT* gene IAs or the *HTT* CAG repeats size are associated with the risk for developing α-synucleinopathies and to elucidate whether they can behave as modulators of the phenotype, by analyzing a case-control series that included patients with either clinical or neuropathological diagnosis of α-synucleinopathy.

2-Methods

Subjects

This is a multicenter study involving subjects recruited from the following out-patient clinics: Hospital Universitario Central de Asturias (Oviedo, Spain), Hospital Universitario de Cabueñes (Gijón, Spain), Hospital Universitario Marqués de Valdecilla (Santander, Spain), Hospital Universitario Virgen del Rocío (Sevilla, Spain), Hospital Clínic i Provincial (Barcelona, Spain), Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), University Hospital Mutua de Terrassa (Terrassa, Barcelona, Spain), ACE Alzheimer Center (Barcelona, Spain) as well as the samples with neuropathological confirmation which came from the Neurological Tissue Bank of the Hospital Clinic-IDIBAPS (Barcelona, Spain). Biobank Valdecilla (PT17/0015/0019, Santander, Spain), the SSPA (PT17/0015/0041) Biobank and the Principado de Asturias BioBank (Oviedo, Spain, PT17/0015/0023). Diagnosis of PD, PD dementia (PDD), DLB and MSA were performed according to the standard research criteria.[8-11] Neuropathological diagnosis of the other α-synucleinopathies was performed according to standard criteria.[12-15] Gender, age at onset, the age of the last visit and the age of death were collected. All the participants or legal representatives gave written informed consent to participate in the study which was approved by the Ethical Committees of Hospital Universitario Central de Asturias and collaborating centers.

Genetic analysis

Genomic DNA was isolated either from peripheral blood leukocytes or from brain tissue (mostly cerebellum, frontal cortex or caudate nucleus) following standard procedures. Specifically, DNA from all subjects with definite MSA was isolated from cerebellum. *HTT* gene CAG repeat number was analyzed by a polymerase chain reaction with 50-fluorescence labeled primers.[16] The number of repeats was determined by capillary electrophoresis using an ABI 3130X automated DNA sequencer and the *GeneMapper* version 4.0 software (Applied Biosystems, Foster City, CA, USA). To provide size standards, several samples with different *HTT* CAG allele length were sequenced as positive controls. In individuals with *HTT* CAG expanded alleles, Sanger sequencing was performed in order to analyze the loss of CAA interruption which is associated with early onset HD and it is particularly relevant to individuals carrying alleles in the reduced penetrance range (ie, CAG 36 -39).[6,17] In terms of interpretation of *HTT* CAG repeats, the shorter allele was called “allele 1” and the longer allele “allele 2”

(*Supplementary Figure 1). APOE*-Ɛ isoforms (SNPs rs7412 + rs4293589) were genotyped with a real-time PCR *Taqman* ® custom assay (Applied Biosystems, Foster City, CA, USA) as described.[18]

Neuropathological study

We study whether subjects with intermediate *HTT* CAG copies had brain polyglutamine deposits in 5 IAs carriers with pathologically confirmed MS. Two MSA subjects with normal range *HTT* CAG sizes were used as controls. Five micrometer formalin-fixed, paraffin embedded sections from motor cortex, caudate, putamen, globus pallidus, nucleus basalis, pons and cerebellum were stained with polyglutamine (Clone 5TF1-1C2, 1/5000, Mouse, Milipore). A Leica Bond Max platform was used, with 20 minutes of pretreatment with Leica ER1 (citrate based) and 60 minutes of primary antibody incubation. Neuropathological assessment was performed by LMP and IA blinded to the number of *HTT* CAG repeats.

Statistical analyses

In order to compare the frequency of the IAs of *HTT* among groups, Chi-square and Fisher's tests were performed. To analyze the influence of expanded IAs on the disease onset, *Kruskal-Wallis* test was performed, with their corresponding Holm adjustment, after verifying by means of the *Kolmogorov-Smirnov* test when the variable did not follow a normal distribution. To determine the relationship between the HTT CAG IAs and the survival probability a Kaplan-Meier method and the long-rank test were used. Survival was calculated as time from symptom onset to death from any cause (outcome= 1) or censoring date (outcome= 0). Categorical variables were described in frequencies and percentages. Mean and standard deviation were used for the analysis of continuous variables. To assess the effect of predictors on specific neurological conditions, multivariate logistic regression models were created including all the independent factors available.

Multiple comparison corrections were made by pairwise comparisons for categorical variables (normal, intermediate or expanded *HTT* CAG allele, death rate and gender) and Dunn's Multiple Comparison Test for quantitative variables (disease group, *HTT* CAG repeat number. age at onset, age at last visit/death and disease duration). Statistical analysis was performed using R statistical software (version 4.0.5) and SPSS Statistics (version 19).

<u>**Data availability statement**</u>

The data that support the findings of this study are available from the corresponding author upon reasonable request.

<u>***3-Results***</u>

Subjects

HTT gene CAG repeats were analyzed in 2702 subjects. Among the 1649 patients, 370 had a diagnosis of DLB, 123 of MSA, 132 of PDD and 1024 of PD, including 610 PD samples previously published[6]. Forty-four patients underwent neuropathological postmortem exam (*Table 1).* The control group comprised 1053 unrelated healthy subjects without any neurological condition nor family history of neurological diseases *(Supplementary Figure 2*)

There was a male predominance among the PD group (57.7%) compared with controls (46.5%; $p<.001$; OR :1.57 (CI 95%;1.31-1-87), whereas the gender was equally balanced among the other groups. Age at onset was statistically different among all disease groups ($p<.05$), by having the MSA patients the earliest disease onset. Besides, males started the disease earlier than females in both, DLB ($p<.05$) and PD ($p<.05$) groups. PD showed a longer disease duration (10.03±8.19, $p<.001$) compared with the other groups. The MSA showed earlier age at death than that of the other disease groups (66.11±9.79, $p<.001$) (*Table 1*)

The *APOE*-Ɛ4 allele frequency was higher in the DLB cohort than in controls [35.3% vs 18.0%, respectively; $p<.001$; OR:2.49 (CI 95%; 1.88-3.29); *Table 2*]. No other differences for the *APOE*-Ɛ4 allele frequency were observed when comparing other patient groups with controls.

***HTT* low-penetrance (36-39) repeat expansions in patients with α-synucleinopathies**

Three PD patients (3/1010 = 0.30%) carried low penetrance-pathological CAG expanded *HTT* alleles (36-39 repeat range). Two 75 and 66 years-old female healthy controls (0.19%; 2/1039) carried low penetrance-pathological CAG expanded *HTT* alleles of 37 and 39 repeats, respectively. The frequency of low penetrance expanded alleles was not statistically significant between PD patients and controls (p= 0.6829; OR (CI95%):1.54 (0.18-18.52)). No carriers of

HTT alleles in the 36-39 range were observed among the DLB, MSA or the PDD groups. Loss of CAA interruptions were not detected after Sanger sequencing in the carriers of 36-39 range CAG *HTT* alleles.

The low penetrance PD carriers, they all fulfilled standard clinical criteria for PD and one of them also had a mild cognitive impairment which was cognitively stable until the last visit (follow-up of 4 years). One of them showed motor fluctuations with prominent leg dystonia. None of them had chorea, other additional movement disorders nor dementia. The diagnosis of PD was supported in two of them by the brain 123-Ioflupane SPECT (*DATscan*). No family history of HD was reported for any of their relatives. Detailed clinical features are depicted in *Table 3.*

HTT Intermediate alleles in α-synucleinopathies

DLB, PDD and PD clinical groups showed no differences in the frequency of IAs carriers compared with controls, although in the MSA clinical group, IAs carrier's frequency was slightly higher than controls (6.3% vs 3.9%; p-value >.1; OR (CI 95%): 1.64 (0.49-4.34). When considering the patients with definite MSA, the IAs frequency was statistically significantly higher than in controls (14.3% vs 3.9 %; p<.05; OR (CI 95%): 4.05 (1.17-11.31). However, considering all the patients with of MSA, the IAs frequency was not significantly different from controls (8.8% in MSA vs 3.9% in healthy controls, p>.05; OR (CI 95%):2.34 (1.01-4.92)) (*Table 4)*.

No differences in the mean age at onset or survival probability between IAs carriers and non carriers were observed in any of the groups (*Supplementary Figure 3)*. Regarding gender distribution, there was a significantly higher frequency of females carrying IAs compared with males in the DLB cohort [8.5% vs 3.2% respectively; p<.05; OR (CI 95%): 2.82 (0.99-9.18); *Supplementary Table 1]*.

HTT CAG size effect on α-synucleinopathies

Considering the *HTT* size, DLB (20.27 ± 3.37; p <.01; CI 95% : (0.179-1.01)) and PD patients (20.14 ± 3.40; p <.01; CI95%: (0.168-0.81)) had a higher mean of repeats in the HTT allele 2 than controls (19.67 ± 3.41).

Multivariate logistic models used to identify if any independent factors were related to the risk of certain α-synucleinopathies were run. The first model (Cox-Snell's R^2 =.014) that included the number of CAG repeats in *HTT,* alleles 1 (shorter allele) and 2 (longer allele), showed an effect of the HTT CAG allele 2 size to develop DLB (β =.075; p =.002) and PD (β =.060; p =.004). The second model (Cox-Snell's R^2 =.074) that included the size of *HTT* CAG alleles 1 and 2, APOE status (APOE-Ɛ4 carriers vs non carriers) and gender, showed that being male (β =.561; p <.001), APOE-Ɛ4 (β = 1.025; p <.001) and *HTT* allele 2 size (β =.109; p <.001) increased the risk for DLB. In addition, this second model showed an effect of being male (β =.814; p <.001) and HTT allele 2 size (β =.060; p =.027) to develop PD as well as an additional effect of *HTT* allele 2 size (β =.089; p =.028) to develop PDD. No other regression models displayed additional effects of the independent variables in the other groups.

Neuropathological analysis of *HTT CAG i*ntermediate allele carriers

Post-mortem brain analysis of 5 MSA patients carrying IAs, showed that 3 subjects who had a MSA parkinsonian type (MSA-P) presented striatonigral degeneration stages II/IIIa (*Table 5*). Interestingly, in the clinical group 4 out 5 IAs carriers also showed a MSA-P presentation.

We observed polyglutamine staining in isolated neurons in two MSA IAs carriers. Subject MSA-2 displayed a nuclear granular staining in isolated neurons in the pontine nuclei and in the pontine tegmentum. On the other hand, MSA-4 brain showed granular to coarse cytoplasmic inclusions in isolated small neurons, the putamen and caudate nuclei. Importantly, these two subjects carried the longest *HTT* IAs CAG alleles (32 CAG repeats). Cytoplasmic inclusions were not observed in the MSA control group with normal range *HTT* CAG alleles (n=2), nor in other MSA IAs carriers (n=4) (*Supplementary Figure 4; Supplementary Table 2*).

4-Discussion

In this study, we investigated whether the expanded *HTT* CAG alleles can lead to an additional risk to develop α-synucleinopathies. With regards to the frequency of pathogenic *HTT* CAG repeat expansions in the sample, we identified three PD patients who carried pathogenic low-penetrance *HTT* CAG repeat expansions representing 0.30% of the PD cohort. However, we found two healthy controls who were also carriers of reduced penetrance *HTT* CAG expansions (0.19%) being the differences of frequency distribution not statistically significant. None of the DLB or MSA carried pathogenic *HTT* CAG expansions.

A recent study in FTD/ALS cohorts showed a frequency of low-penetrance (36-39) HTT repeat expansions of 0.20, 0.19% and 0.22% in FTD/ALS, DLB and healthy controls respectively, suggesting that low-penetrance *HTT* repeat expansions have no effect in TDP-43 proteinopathies or α-synucleinopathies[5]. However, Dewan et al.[5] analyzed the brains from two patients with FTD/ALS carrying pathological *HTT* CAG repeat expansions and found huntingtin and ubiquitin-positive aggregates in the frontal cortex with a different distribution from the standard brain pathology found in HD.

Unfortunately, we do not have available other large studies that have assessed the frequency of low-penetrance *HTT* CAG expansions in Spain in order to confirm if these control frequencies reflect accurately the real population frequency.,[20] Considering the frequencies of low-penetrance *HTT* CAG repeat expansions in PD patients and controls , the comparison suggests that there is no enrichment of the expansion in our PD sample.

However, knowing that the chance of subjects carrying low-penetrance *HTT* expansions of being symptomatic for HD at 75 years of age is about 60%[21] and taking into account that our PD carriers had an average age of 69.6 years, it could be expected to find HD signs in some of them. Since our PD carriers did not show any atypical PD signs either, these data suggest that if low-penetrance pathogenic CAG repeat expansions in *HTT* gene, have any effect on the clinical outcomes of the PD in some cases, we could not detect such effect, given the reduced number of carriers available. Future neuropathological studies of brains from PD carriers of low-penetrance pathogenic CAG repeat expansions in *HTT* gene will elucidate their role in PD.

In addition, we found a clear increase of *HTT* CAG repeat size among DLB and PD groups that was influenced by the male gender and also by the presence of *APOE4* allele in DLB patients. These results suggest a role of *HTT* CAG repeats in the risk of intracellular α-synucleinopathies and it seems that their effect can be associated with other genetic factors such as the gender and the *APOE4* allele.

With regards to the analysis of *HTT* CAG IAs, we found an increased frequency of *HTT* IAs in the MSA group compared with controls (8.8% vs 3.8, respectively). This difference was statistically significant in the MSA group with neuropathological confirmation that remained increased when adding the group with clinical diagnosis of MSA. The lack of association in the latter can be owed to the clinical misdiagnosis with other neurological disorders, which has been reported to be up to 18.8% in MSA.[22] The fact that the majority of MSA patients with IAs mostly presented the MSA parkinsonian subtype (MSA-P) suggests that the main source of misdiagnosis could be PD.

In case, this association of MSA risk with an increase of *HTT* CAG IAs is confirmed in other series, it can highlight some clues of the etiology of such devastating α-synucleinopathy since no other strong genetic factors have been associated with MSA to date.[23]

The brain exam of five MSA *HTT* CAG IAs carriers was studied histologically to detect the polyglutamine chains in the proteins originating from the expanded alleles. We observed that two MSA *HTT* GAG IAs carriers with 32 CAG copies showed isolated positivity, in pons and basal nuclei, which are two critical structures in the neurodegeneration of MSA. However, the paucity of polyglutamine inclusions did not allow to perform solid topographical correlations. Nevertheless, polyglutamine expansions were not seen in the MSA control group, nor in other MSA IAs *HTT* carriers with less than 32 CAG repeats, hence the observed results, albeit limited, are remarkable. Interestingly, Dewan et al[5] also found isolated polyQ inclusions in TDP-43 ALS brains carrying *HTT* expanded alleles across different brain regions, mainly prefrontal and motor cortex, caudate nucleus and striatum. Interestingly, the location of the polyQ inclusions found bt them is partially different from that of the brains of the MSA IAs *HTT* carriers of the present study, but still suggest that the striatum could be a common target region to polyQ aggregation in both HD and non-HD clinical phenotype.

In the last few years, several studies assessed the potential pathogenic effect of IAs in several genes on different neurodegenerative diseases. Expansions of the CAG repeats in the *ATXN2* gene have been associated with increased risk for ALS and could be a FTD phenotype modifier and i *ATXN1* IAs have been proposed as a risk factor in ALS.[24,25] In a previous multicentric study, we identified a significant association of *HTT* CAG IAs with disease risk for FTD and a significant increase of *ATXN2* IAs in AD patients compared to controls. In addition, we also found an increased frequency of *HTT* CAG IAS in behavioral FTD variant (bvFTD) and PNFA[7]. These data suggest that IA might play a wide role in different neurodegenerative diseases. Many neurodegenerative diseases accumulate different protein aggregates in the brain, such as amyloid, tau, a-syn, or TDP-43,[26-30] although, very often different protein aggregates coexist within the same brain. The brain aggregates in α-synucleinopathies (PD, PDD, DLB and MSA) consist primarily of a-syn protein whereas the main component of HD aggregates is huntingtin (Htt). The brain regions initially affected by these aggregates and the clinical symptoms are also different among the different neurodegenerative conditions. In addition, much data supports the interactions between mutant Htt and other pathological proteins. A crosstalk between Htt aggregation and a-syn has been speculated, suggesting a modifying effect of a-syn on Htt aggregation.[31] In addition, increased a-syn putaminal aggregation and a-syn serum levels have been reported in HD patients[21]. In addition, it has been shown that a-syn and Htt can interact and co-aggregate in cell and animal models and human brains.[32,33,34,35] These data suggest that *HTT* CAG IAs can behave as triggers of the aggregation not only of Htt but also of other pathological proteins as well and this could explain a *HTT* IAs' role as modifying genetic factors although their real effect on the phenotypic expression has to be elucidate.

Our results, in line with previous works, pointed to a link between *HTT* IAs and other non HD brain proteinopathies and also support the hypothesis that they can share common pathways. It might be of utmost importance to know how IAs in the *HTT* gene contribute to neurodegeneration acting as a genetic modifier.

We are aware that our study has several limitations, since the sample size for some of neuropathological cohorts was small, suggesting the need of replication of additional series to confirm these results. Specifically, the neuropathological findings of MSA with IAs, should be tested and replicated in larger samples that allows to perform topographical correlations. Thus, further neuropathological studies are needed to analyze whether polyQ deposits are constantly present in a considerable percentage of brains of IAs carriers with MSA. Future biochemical studies are needed to elucidate whether IAs *HTT* CAG or more expanded alleles can trigger not only the Htt aggregation, but also of other pathological proteins such as a-syn as well, and this explain their genetic risk.

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6- Author roles

Name	***Location***	***Role***	***Contribution***
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Ignacio Álvarez	University Hospital Mutua Terrassa	First Author	Analyzed and interpreted the data; drafted the manuscript for intellectual content
Irene Rosas	Hospital Universitario Central de Asturias	Author	Execution (genetic data) Critical revision of the manuscript
Manuel Menéndez-González	Hospital Universitario Central de Asturias	Author	Execution (clinical data) Critical revision of the manuscript
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Victoria Álvarez	Hospital Universitario Central de Asturias	Corresponding author	Design and conceptualized study; interpreted the data. Execution (genetics) of the project, statistical analysis (design and critical analysis) and writing of the first draft of the manuscript.
Pau Pastor	Hospital Germans Trias i Pujol.	Last author	Design and conceptualized study; interpreted the data. Execution (clinical assessments) of the project and writing of the draft of the manuscript.

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Figure legends:

Supplementary Figure1- *Representative TP-PCR electropherograms showing HTT normal, intermediate and expanded CAG alleles.*

Supplementary Figure 2 *Distribution of HTT CAG alleles across the sample. DLB: dementia with Lewy bodies; MSA: Multisystem atrophy; PD: Parkinson disease; PDD: Parkinson´ disease dementia; IAs: intermediate HTT CAG alleles.*

Supplementary Figure 3- *A) Age at onset carriers and non carriers of IAs in the different clinical groups. B) Survival analysis in carriers and non carriers of IAs, PDD was not included because all data are censored. DLB: dementia with Lewy bodies; MSA: Multisystem atrophy; PD: Parkinson disease; PDD: Parkinson´ disease dementia; IAs: intermediate HTT CAG alleles.*

Supplementary Figure 4- *Polyglutamine immunohistochemistry. A and B: two pontine neurons with diffuse nuclear positivity in subject MSA-2. In A, a focal cytoplasmic positivity is also noted. C putamen and D caudate nucleus of case MSA-4. Isolated cells, suggestive of neurons, showed focal cytoplasmic positivity. All images 400x, scale bar 20 µm.*

Intermediate and expanded *HTT* alleles and the risk for α-synucleinopathies

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RELEVANT CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES:
The authors report no conflicts of interest related to this work.

Abstract

Background: Previous studies suggest a link between CAG repeat number in the *HTT* gene and non-Huntington neurodegenerative diseases.

Objective: To analyze whether expanded HTT CAG alleles and/or their size are associated with the risk for developing α-synucleinopathies or their behavior as modulators of the phenotype.

Methods: We genotyped the *HTT* gene CAG repeat number and APOE-Ɛ isoforms in a case-control series including patients with either clinical or neuropathological diagnosis of α-synucleinopathy.

Results: We identified three PD patients (0.30%) and two healthy controls (0.19%) carrying low-penetrance *HTT* repeat expansions whereas none of the DLB or MSA patients carried pathogenic *HTT* expansions. In addition, a clear increase of the number of *HTT* CAG repeats was found among DLB and PD groups influenced by the male gender and also by the *APOE4* allele among DLB patients. *HTT* Intermediate alleles' (IAs) distribution frequency was increased in the MSA group compared with controls (8.8% vs 3.9%, respectively). These differences were indeed statistically significant in the MSA group with neuropathological confirmation. Two MSA *HTT* CAG IAs carriers with 32 *HTT* CAG repeats showed isolated polyQ inclusions in pons and basal nuclei, which are two critical structures in the neurodegeneration of MSA.

Conclusions: Our results pointed to a link between, *HTT* CAG number, *HTT* IAs and expanded *HTT* CAG repeats with other non -HD brain pathology and support the hypothesis that they can share common neurodegenerative pathways.

1-Introduction

The most frequent α-synucleinopathies, which are characterized by α-synuclein (α-syn) brain aggregates are Parkinson disease (PD), dementia with Lewy bodies (DLB) and multisystem atrophy (MSA). MSA is characterized by glial cytoplasmic α-synuclein (a-syn) inclusions, whereas in DLB and PD by intraneuronal Lewy bodies.[1] The brain exam from patients with α-synucleinopathies show very often concurrence of many other neurodegenerative diseases with tau or amyloid deposits.[2,3] Hence, it is possible that α-synucleinopathies also share other neuropathogenic mechanisms related to α-syn pathways that can be reflected in their genetic architecture.

Huntington disease (HD) is an inherited neurodegenerative disease linked to a CAG repeat expansion at the *Huntingtin* gene *(HTT*; MIM:613004). CAG *HTT* allele expansions above 36 can cause HD and above 41 are fully penetrant, whereas CAG *HTT* intermediate alleles (IAs; range 27-35) are unstable and are prone to increase their length into a pathological range in the offspring.[4] Recently, a putative etiologic role of the CAG repeats in the *HTT* gene has been proposed for non-Huntington neurodegenerative diseases as shown in an analysis of *HTT* gene CAG repeats in a large cohort of frontotemporal dementia/amyotrophic lateral sclerosis (FTD/ALS) and DLB, that showed fully-penetrant pathological *HTT* CAG gene expansions in 0.12-0.14% of FTD/ALS cohorts but not among DLB patients.[5] In addition, previous studies from our group found that IAs in *HTT* can have a role in AD risk[6] and CAG IAs in the *ATXN1, ATXN2* and *HTT* genes showed a variable frequency in different FTD subtypes.[7] These results further pointed towards a link between IAs and tauopathies, suggesting an involvement of *HTT* CAG expanded alleles in other neurodegenerative diseases.

To unravel the genetic architecture of α-synucleinopathies is critical for the design of clinical trials and will contribute to develop new drugs and disease modifying interventions. Thus, the main objective of this study is to determine whether the *HTT* gene IAs or the *HTT* CAG repeats size are associated with the risk for developing α-synucleinopathies and to elucidate whether they can behave as modulators of the phenotype, by analyzing a case-control series that included patients with either clinical or neuropathological diagnosis of α-synucleinopathy.

2-Methods

Subjects

This is a multicenter study involving subjects recruited from the following out-patient clinics: Hospital Universitario Central de Asturias (Oviedo, Spain), Hospital Universitario de Cabueñes (Gijón, Spain), Hospital Universitario Marqués de Valdecilla (Santander, Spain), Hospital Universitario Virgen del Rocío (Sevilla, Spain), Hospital Clínic i Provincial (Barcelona, Spain), Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), University Hospital Mutua de Terrassa (Terrassa, Barcelona, Spain), ACE Alzheimer Center (Barcelona, Spain) as well as the samples with neuropathological confirmation which came from the Neurological Tissue Bank of the Hospital Clinic-IDIBAPS (Barcelona, Spain). Biobank Valdecilla (PT17/0015/0019, Santander, Spain), the SSPA (PT17/0015/0041) Biobank and the Principado de Asturias BioBank (Oviedo, Spain, PT17/0015/0023). Diagnosis of PD, PD dementia (PDD), DLB and MSA were performed according to the standard research criteria.[8-11] Neuropathological diagnosis of the other α-synucleinopathies was performed according to standard criteria.[12-15] Gender, age at onset, the age of the last visit and the age of death were collected. All the participants or legal representatives gave written informed consent to participate in the study which was approved by the Ethical Committees of Hospital Universitario Central de Asturias and collaborating centers.

Genetic analysis

Genomic DNA was isolated either from peripheral blood leukocytes or from brain tissue (mostly cerebellum, frontal cortex or caudate nucleus) following standard procedures. Specifically, DNA from all subjects with definite MSA was isolated from cerebellum. *HTT* gene CAG repeat number was analyzed by a polymerase chain reaction with 50-fluorescence labeled primers.[16] The number of repeats was determined by capillary electrophoresis using an ABI 3130X automated DNA sequencer and the *GeneMapper* version 4.0 software (Applied Biosystems, Foster City, CA, USA). To provide size standards, several samples with different *HTT* CAG allele length were sequenced as positive controls. In individuals with *HTT* CAG expanded alleles, Sanger sequencing was performed in order to analyze the loss of CAA interruption which is associated with early onset HD and it is particularly relevant to individuals carrying alleles in the reduced penetrance range (ie, CAG 36 -39).[6,17] In terms of interpretation of *HTT* CAG repeats, the shorter allele was called “allele 1” and the longer allele “allele 2”

(*Supplementary Figure 1*). *APOE*-Ɛ isoforms (SNPs rs7412 + rs4293589) were genotyped with a real-time PCR *Taqman* ® custom assay (Applied Biosystems, Foster City, CA, USA) as described.[18]

Neuropathological study

We study whether subjects with intermediate *HTT* CAG copies had brain polyglutamine deposits in 5 IAs carriers with pathologically confirmed MS. Two MSA subjects with normal range *HTT* CAG sizes were used as controls. Five micrometer formalin-fixed, paraffin embedded sections from motor cortex, caudate, putamen, globus pallidus, nucleus basalis, pons and cerebellum were stained with polyglutamine (Clone 5TF1-1C2, 1/5000, Mouse, Milipore). A Leica Bond Max platform was used, with 20 minutes of pretreatment with Leica ER1 (citrate based) and 60 minutes of primary antibody incubation. Neuropathological assessment was performed by LMP and IA blinded to the number of *HTT* CAG repeats.

Statistical analyses

In order to compare the frequency of the IAs of *HTT* among groups, Chi-square and Fisher's tests were performed. To analyze the influence of expanded IAs on the disease onset, *Kruskal-Wallis* test was performed, with their corresponding Holm adjustment, after verifying by means of the *Kolmogorov-Smirnov* test when the variable did not follow a normal distribution. To determine the relationship between the HTT CAG IAs and the survival probability a Kaplan-Meier method and the long-rank test were used. Survival was calculated as time from symptom onset to death from any cause (outcome= 1) or censoring date (outcome= 0). Categorical variables were described in frequencies and percentages. Mean and standard deviation were used for the analysis of continuous variables. To assess the effect of predictors on specific neurological conditions, multivariate logistic regression models were created including all the independent factors available.

Multiple comparison corrections were made by pairwise comparisons for categorical variables (normal, intermediate or expanded *HTT* CAG allele, death rate and gender) and Dunn's Multiple Comparison Test for quantitative variables (disease group, *HTT* CAG repeat number. age at onset, age at last visit/death and disease duration). Statistical analysis was performed using R statistical software (version 4.0.5) and SPSS Statistics (version 19).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3-Results

Subjects

HTT gene CAG repeats were analyzed in 2702 subjects. Among the 1649 patients, 370 had a diagnosis of DLB, 123 of MSA, 132 of PDD and 1024 of PD, including 610 PD samples previously published[6]. Forty-four patients underwent neuropathological postmortem exam (*Table 1).* The control group comprised 1053 unrelated healthy subjects without any neurological condition nor family history of neurological diseases *(Supplementary Figure 2*)

There was a male predominance among the PD group (57.7%) compared with controls (46.5%; p<.001; OR :1.57 (CI 95%;1.31-1-87), whereas the gender was equally balanced among the other groups. Age at onset was statistically different among all disease groups (p<.05), by having the MSA patients the earliest disease onset. Besides, males started the disease earlier than females in both, DLB (p<.05) and PD (p<.05) groups. PD showed a longer disease duration (10.03±8.19, p<.001) compared with the other groups. The MSA showed earlier age at death than that of the other disease groups (66.11±9.79, p<.001) (*Table 1*)

The *APOE*-Ɛ4 allele frequency was higher in the DLB cohort than in controls [35.3% vs 18.0%, respectively; p<.001; OR:2.49 (CI 95%; 1.88-3.29); *Table 2*]. No other differences for the *APOE*-Ɛ4 allele frequency were observed when comparing other patient groups with controls.

HTT **low-penetrance (36-39) repeat expansions in patients with α-synucleinopathies**

Three PD patients (3/1010 = 0.30%) carried low penetrance-pathological CAG expanded *HTT* alleles (36-39 repeat range). Two 75 and 66 years-old female healthy controls (0.19%; 2/1039) carried low penetrance-pathological CAG expanded *HTT* alleles of 37 and 39 repeats, respectively. The frequency of low penetrance expanded alleles was not statistically significant between PD patients and controls (p= 0.6829; OR (CI95%):1.54 (0.18-18.52)). No carriers of

HTT alleles in the 36-39 range were observed among the DLB, MSA or the PDD groups. Loss of CAA interruptions were not detected after Sanger sequencing in the carriers of 36-39 range CAG *HTT* alleles.

The low penetrance PD carriers, they all fulfilled standard clinical criteria for PD and one of them also had a mild cognitive impairment which was cognitively stable until the last visit (follow-up of 4 years). One of them showed motor fluctuations with prominent leg dystonia. None of them had chorea, other additional movement disorders nor dementia. The diagnosis of PD was supported in two of them by the brain 123-Ioflupane SPECT (*DATscan*). No family history of HD was reported for any of their relatives. Detailed clinical features are depicted in *Table 3.*

HTT Intermediate alleles in α-synucleinopathies

DLB, PDD and PD clinical groups showed no differences in the frequency of IAs carriers compared with controls, although in the MSA clinical group, IAs carrier's frequency was slightly higher than controls (6.3% vs 3.9%; p-value >.1; OR (CI 95%): 1.64 (0.49-4.34). When considering the patients with definite MSA, the IAs frequency was statistically significantly higher than in controls (14.3% vs 3.9 %; p<.05; OR (CI 95%): 4.05 (1.17-11.31). However, considering all the patients with of MSA, the IAs frequency was not significantly different from controls (8.8% in MSA vs 3.9% in healthy controls, p>.05; OR (CI 95%):2.34 (1.01-4.92)) (*Table 4)*.

No differences in the mean age at onset or survival probability between IAs carriers and non carriers were observed in any of the groups (*Supplementary Figure 3)*. Regarding gender distribution, there was a significantly higher frequency of females carrying IAs compared with males in the DLB cohort [8.5% vs 3.2% respectively; p<.05; OR (CI 95%): 2.82 (0.99-9.18); *Supplementary Table 1]*.

HTT CAG size effect on α-synucleinopathies

Considering the *HTT* size, DLB (20.27 ± 3.37; p <.01; CI 95% : (0.179-1.01)) and PD patients (20.14 ± 3.40; p <.01; CI95%: (0.168-0.81)) had a higher mean of repeats in the HTT allele 2 than controls (19.67 ± 3.41).

Multivariate logistic models used to identify if any independent factors were related to the risk of certain α-synucleinopathies were run. The first model (Cox-Snell's R^2 =.014) that included the number of CAG repeats in *HTT,* alleles 1 (shorter allele) and 2 (longer allele), showed an effect of the HTT CAG allele 2 size to develop DLB (β =.075; p =.002) and PD (β =.060; p =.004). The second model (Cox-Snell's R^2 =.074) that included the size of *HTT* CAG alleles 1 and 2, APOE status (APOE-Ɛ4 carriers vs non carriers) and gender, showed that being male (β =.561; p <.001), APOE-Ɛ4 (β = 1.025; p <.001) and *HTT* allele 2 size (β =.109; p <.001) increased the risk for DLB. In addition, this second model showed an effect of being male (β =.814; p <.001) and HTT allele 2 size (β =.060; p =.027) to develop PD as well as an additional effect of *HTT* allele 2 size (β =.089; p =.028) to develop PDD. No other regression models displayed additional effects of the independent variables in the other groups.

Neuropathological analysis of *HTT CAG i*ntermediate allele carriers

Post-mortem brain analysis of 5 MSA patients carrying IAs, showed that 3 subjects who had a MSA parkinsonian type (MSA-P) presented striatonigral degeneration stages II/IIIa (*Table 5*). Interestingly, in the clinical group 4 out 5 IAs carriers also showed a MSA-P presentation.

We observed polyglutamine staining in isolated neurons in two MSA IAs carriers. Subject MSA-2 displayed a nuclear granular staining in isolated neurons in the pontine nuclei and in the pontine tegmentum. On the other hand, MSA-4 brain showed granular to coarse cytoplasmic inclusions in isolated small neurons, the putamen and caudate nuclei. Importantly, these two subjects carried the longest *HTT* IAs CAG alleles (32 CAG repeats). Cytoplasmic inclusions were not observed in the MSA control group with normal range *HTT* CAG alleles (n=2), nor in other MSA IAs carriers (n=4) (*Supplementary Figure 4; Supplementary Table 2*).

4-Discussion

In this study, we investigated whether the expanded *HTT* CAG alleles can lead to an additional risk to develop α-synucleinopathies. With regards to the frequency of pathogenic *HTT* CAG repeat expansions in the sample, we identified three PD patients who carried pathogenic low-penetrance *HTT* CAG repeat expansions representing 0.30% of the PD cohort. However, we found two healthy controls who were also carriers of reduced penetrance *HTT* CAG expansions (0.19%) being the differences of frequency distribution not statistically significant. None of the DLB or MSA carried pathogenic *HTT* CAG expansions.

A recent study in FTD/ALS cohorts showed a frequency of low-penetrance (36-39) HTT repeat expansions of 0.20, 0.19% and 0.22% in FTD/ALS, DLB and healthy controls respectively, suggesting that low-penetrance *HTT* repeat expansions have no effect in TDP-43 proteinopathies or α-synucleinopathies[5]. However, Dewan et al.[5] analyzed the brains from two patients with FTD/ALS carrying pathological *HTT* CAG repeat expansions and found huntingtin and ubiquitin-positive aggregates in the frontal cortex with a different distribution from the standard brain pathology found in HD.

Unfortunately, we do not have available other large studies that have assessed the frequency of low-penetrance *HTT* CAG expansions in Spain in order to confirm if these control frequencies reflect accurately the real population frequency.,[20] Considering the frequencies of low-penetrance *HTT* CAG repeat expansions in PD patients and controls , the comparison suggests that there is no enrichment of the expansion in our PD sample.

However, knowing that the chance of subjects carrying low-penetrance *HTT* expansions of being symptomatic for HD at 75 years of age is about 60%[21] and taking into account that our PD carriers had an average age of 69.6 years, it could be expected to find HD signs in some of them. Since our PD carriers did not show any atypical PD signs either, these data suggest that if low-penetrance pathogenic CAG repeat expansions in *HTT* gene, have any effect on the clinical outcomes of the PD in some cases, we could not detect such effect, given the reduced number of carriers available. Future neuropathological studies of brains from PD carriers of low-penetrance pathogenic CAG repeat expansions in *HTT* gene will elucidate their role in PD.

In addition, we found a clear increase of *HTT* CAG repeat size among DLB and PD groups that was influenced by the male gender and also by the presence of *APOE4* allele in DLB patients. These results suggest a role of *HTT* CAG repeats in the risk of intracellular α-synucleinopathies and it seems that their effect can be associated with other genetic factors such as the gender and the *APOE4* allele.

With regards to the analysis of *HTT* CAG IAs, we found an increased frequency of *HTT* IAs in the MSA group compared with controls (8.8% vs 3.8, respectively). This difference was statistically significant in the MSA group with neuropathological confirmation that remained increased when adding the group with clinical diagnosis of MSA. The lack of association in the latter can be owed to the clinical misdiagnosis with other neurological disorders, which has been reported to be up to 18.8% in MSA.[22] The fact that the majority of MSA patients with IAs mostly presented the MSA parkinsonian subtype (MSA-P) suggests that the main source of misdiagnosis could be PD.

In case, this association of MSA risk with an increase of *HTT* CAG IAs is confirmed in other series, it can highlight some clues of the etiology of such devastating α-synucleinopathy since no other strong genetic factors have been associated with MSA to date.[23]

The brain exam of five MSA *HTT* CAG IAs carriers was studied histologically to detect the polyglutamine chains in the proteins originating from the expanded alleles. We observed that two MSA *HTT* GAG IAs carriers with 32 CAG copies showed isolated positivity, in pons and basal nuclei, which are two critical structures in the neurodegeneration of MSA. However, the paucity of polyglutamine inclusions did not allow to perform solid topographical correlations. Nevertheless, polyglutamine expansions were not seen in the MSA control group, nor in other MSA IAs *HTT* carriers with less than 32 CAG repeats, hence the observed results, albeit limited, are remarkable. Interestingly, Dewan et al[5] also found isolated polyQ inclusions in TDP-43 ALS brains carrying *HTT* expanded alleles across different brain regions, mainly prefrontal and motor cortex, caudate nucleus and striatum. Interestingly, the location of the polyQ inclusions found bt them is partially different from that of the brains of the MSA IAs *HTT* carriers of the present study, but still suggest that the striatum could be a common target region to polyQ aggregation in both HD and non-HD clinical phenotype.

In the last few years, several studies assessed the potential pathogenic effect of IAs in several genes on different neurodegenerative diseases. Expansions of the CAG repeats in the *ATXN2* gene have been associated with increased risk for ALS and could be a FTD phenotype modifier and i *ATXN1* IAs have been proposed as a risk factor in ALS.[24,25] In a previous multicentric study, we identified a significant association of *HTT* CAG IAs with disease risk for FTD and a significant increase of *ATXN2* IAs in AD patients compared to controls. In addition, we also found an increased frequency of *HTT* CAG IAS in behavioral FTD variant (bvFTD) and PNFA[7]. These data suggest that IA might play a wide role in different neurodegenerative diseases. Many neurodegenerative diseases accumulate different protein aggregates in the brain, such as amyloid, tau, a-syn, or TDP-43,[26-30] although, very often different protein aggregates coexist within the same brain. The brain aggregates in α-synucleinopathies (PD, PDD, DLB and MSA) consist primarily of a-syn protein whereas the main component of HD aggregates is huntingtin (Htt). The brain regions initially affected by these aggregates and the clinical symptoms are also different among the different neurodegenerative conditions. In addition, much data supports the interactions between mutant Htt and other pathological proteins. A crosstalk between Htt aggregation and a-syn has been speculated, suggesting a modifying effect of a-syn on Htt aggregation.[31] In addition, increased a-syn putaminal aggregation and a-syn serum levels have been reported in HD patients[21]. In addition, it has been shown that a-syn and Htt can interact and co-aggregate in cell and animal models and human brains.[32,33,34,35] These data suggest that *HTT* CAG IAs can behave as triggers of the aggregation not only of Htt but also of other pathological proteins as well and this could explain a *HTT* IAs' role as modifying genetic factors although their real effect on the phenotypic expression has to be elucidate.

Our results, in line with previous works, pointed to a link between *HTT* IAs and other non HD brain proteinopathies and also support the hypothesis that they can share common pathways. It might be of utmost importance to know how IAs in the *HTT* gene contribute to neurodegeneration acting as a genetic modifier.

We are aware that our study has several limitations, since the sample size for some of neuropathological cohorts was small, suggesting the need of replication of additional series to confirm these results. Specifically, the neuropathological findings of MSA with IAs, should be tested and replicated in larger samples that allows to perform topographical correlations. Thus, further neuropathological studies are needed to analyze whether polyQ deposits are constantly present in a considerable percentage of brains of IAs carriers with MSA. Future biochemical studies are needed to elucidate whether IAs *HTT* CAG or more expanded alleles can trigger not only the Htt aggregation, but also of other pathological proteins such as a-syn as well, and this explain their genetic risk.

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6- Author roles

Name	Location	Role	Contribution
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Ignacio Álvarez	University Hospital Mutua Terrassa	First Author	Analyzed and interpreted the data; drafted the manuscript for intellectual content
Irene Rosas	Hospital Universitario Central de Asturias	Author	Execution (genetic data) Critical revision of the manuscript
Manuel Menéndez-González	Hospital Universitario Central de Asturias	Author	Execution (clinical data) Critical revision of the manuscript
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Victoria Álvarez	Hospital Universitario Central de Asturias	Corresponding author	Design and conceptualized study; interpreted the data. Execution (genetics) of the project, statistical analysis (design and critical analysis) and writing of the first draft of the manuscript.
Pau Pastor	Hospital Germans Trias i Pujol.	Last author	Design and conceptualized study; interpreted the data. Execution (clinical assessments) of the project and writing of the draft of the manuscript.

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Figure legends:

Supplementary Figure1- *Representative TP-PCR electropherograms showing HTT normal, intermediate and expanded CAG alleles.*

Supplementary Figure 2 *Distribution of HTT CAG alleles across the sample. DLB: dementia with Lewy bodies; MSA: Multisystem atrophy; PD: Parkinson disease; PDD: Parkinson´ disease dementia; IAs: intermediate HTT CAG alleles.*

Supplementary Figure 3- *A) Age at onset carriers and non carriers of IAs in the different clinical groups. B) Survival analysis in carriers and non carriers of IAs, PDD was not included because all data are censored. DLB: dementia with Lewy bodies; MSA: Multisystem atrophy; PD: Parkinson disease; PDD: Parkinson´ disease dementia; IAs: intermediate HTT CAG alleles.*

Supplementary Figure 4- *Polyglutamine immunohistochemistry. A and B: two pontine neurons with diffuse nuclear positivity in subject MSA-2. In A, a focal cytoplasmic positivity is also noted. C putamen and D caudate nucleus of case MSA-4. Isolated cells, suggestive of neurons, showed focal cytoplasmic positivity. All images 400x, scale bar 20 µm.*

Table 1 - Demographic data of the cohorts studied.

All Subjects						
	DLB (n = 370)	***MSA* (n = 123)**	***PDD* (n = 132)**	***PD* (n = 1024)**	**Healthy controls (n = 1053)**	**Sig.**
Gender (Males)	195 (52.7)	55 (44.7)	69 (52.3)	589 (57.7)*	476 (46.5)	<0.001 PD vs controls
Age at onset	72.73 ± 6.82**	59.20±9.53**	68.80±10.46**	61.97±11.95**	NA	<0.05 among all groups
Age at onset (Male)	71.76 ± 7.06***	59.88 ± 10.61	68.65 ± 10.81	61.26 ± 11.72***	NA	<0.05 female vs male in DLB and PD
Age at onset (Female)	73.90 ± 6.34	58.71 ± 8.75	68.98 ± 10.10	62.86 ± 12.20	NA	
Age at death [1]	78.09±5.79	66.11±9.79****	82.12±5.70	77.99±7.77	71.12±7.67 (1)*****	<0.001 MSA vs all other groups <0.01 Controls vs all other groups
Disease duration	5.88±3.88	6.92±4.55	8.12 ±9.41	10.03±8.19****	NA	<0.001 PD vs all other groups
Death rate	44 (12.0)	47 (41.6)*****	17 (18.9)	270 (27.1)******	NA	<0.01 MSA vs all other groups <0.001 PD vs DLB
Neuropathological samples						
	DLB (n = 12)	***MSA* (n = 35)**	***PDD* (n = 0**	***PD* (n = 0)**	**Neurological confirmed controls (n = 24)**	
Gender (Male)	8 (66.7)	15 (42.9)			12 (50.0)	
Age at onset	66.78±7.93******	50.33±7.68			NA	<0.001 MSA vs DLB

Age at death	76.83±7.96*******	64.4±8.66*****			71.17±17.25 (1)	<0.05 DLB vs healthy controls <0.001 MSA vs healthy controls and DLB
Disease duration	9.22±4.27	9.33±2.64			NA	

* $p < 0.001$ compared with controls; ** $p<0.05$ compared with all groups; *** $p<0.05$ comparing males vs females;**** $p<0.001$ compared with all groups; *****$p<0.01$ compared with all groups;****** $p<0.001$ compared with DLB;******* $p<0.05$ compared with controls ; Data presented as mean ± StD or N (%).[1] Controls' age at last visit/death represents the age at DNA donation. DLB: Lewy body Dementia; MSA: Multiple system atrophy; PD: Parkinson disease; PDD: Parkinson disease dementia. NA: not applicable/available.

Table 2 - Frequencies of APOE e4 carriers in the different cohorts.

Pathology	Full cohort			Neuropathological confirmation		
	ApoE4+	p-value	OR (95% CI) vs. controls	ApoE4+	p-value	OR (95% CI) vs. controls
DLB	127 (35.3)	**2.74e-10***	2.49 (1.88-3.29)	1 (16.7)	1.0	0.91 (0.02-8.24)
MSA	21 (19.3)	1.0	1.09 (0.63-1.83)	8 (23.5)	1.0	1.41 (0.54-3.26)
PDD	24 (19.4)	1.0	1.10 (0.65-1.79)			
PD	137 (15.5)	1.0	0.84 (0.65-1.08)			
Controls	176 (18.0)			176 (18.0)		

** $p < 0.005$ compared with controls; OR: Odds ratio; CI: Confidence interval. DLB: Lewy body Dementia; MSA: Multiple system atrophy; PD: Parkinson disease; PDD: Parkinson´ disease dementia.*

Table 3. Clinical characteristics of *HTT* CAG expanded allele carriers.

	Patient 1	**Patient 2**	**Patient 3**
Diagnosis 1	PD	PD	PD
Diagnosis 2	NA	MCI	NA
Age (years)	57	73	79
Age at onset	48	69	74
Disease duration (years)	9	4	5
Main symptom	RUL pain and rest tremor	bilateral UL rest tremor and gait imbalance	Left hand clumsiness and tremor
Other signs/symptoms	RLL dystonia	memory complaints	hypophonia
Chorea	no	no	no
Non-motor symptoms	somnolence, depression and fatigue	no	depression
RBD	no	yes	no
Other medical features	diabetes mellitus	diabetes mellitus, hypercholesterolemia	atrial fibrillation, high blood pressure
Neurological family history	no	mother dementia at 65	no
Familial Huntington disease	no	no	no
UPDRS III	14	9	12
Motor fluctuations	on-off phenomenon	no	no

LED (milligrams)	810	300	750
Brain MRI	NA	MTA score 2	NA
DATscan	no	putaminal hypometabolism	R>L putaminal and caudate hypometabolism
***HTT* gene CAG repeats**	36/22	36/15	37/22

PD = Parkinson disease;RUL= right upper limb;RLL= right lower limb;LED = levodopa-equivalent dose;NA =not available;MCI = mild cognitive impairment;RBD = Rapid eye movement sleep behavior disorder; MTA = medial temporal lobe atrophy; UPDRS III=Unified Parkinson's disease rating scale; UPDRS III=Unified Parkinson's disease rating scale

Table 4 - Frequencies of HTT IAs in the different cohorts.

Pathology (N)	**Full cohort**			**Neuropathological confirmation**				**Clinical diagnosis**			
	HTT IAs N (%)	Corrected p-value	OR (95% CI)	N (%)[1]	*HTT* IAs N (%)	Corrected p-value	OR (95% CI)	N (%)	*HTT* IAs N (%)	Corrected p-value	OR (95% CI)
DLB (n=354)	20 (5.6)	1.0	1.46 (0.80-2.59)	12 (3.4)	1 (8.3)	1.0	2.21 (0.05-15.89)	342 (96.6)	19 (5.6)	1.0	1.43 (0.77-2.56)
MSA (n=114)	10 (8.8)	0.324	2.34 (1.01-4.92)	35 (30.7)	5 (14.3)	**0.033***	4.05 (1.17-11.31)	79 (69.3)	5 (6.3)	1.0	1.64 (0.49-4.34)
PDD (n=129)	5 (3.9)	1.0	0.98 (0.30-2.55)	-	-	-	-	129 (100)	5 (3.9)	1.0	0.98 (0.30-2.55)
PD (n=1010)	42 (4.2)	1.0	1.06 (0.66-1.68)	-	-	-	-	1010 (100)	42 (4.2)	1.0	1.06 (0.66-1.68)
Controls (n=1039)	41 (3.9)			-	-			-	-		

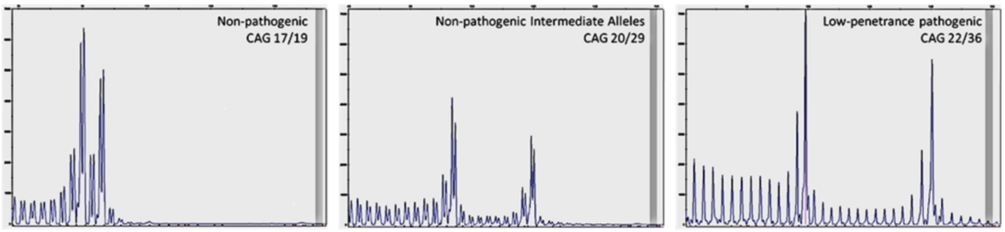
** Statistically significant differences after compared with healthy controls*(p < .05). (1) Percentage of samples of the full cohort. OR: Odds ratio; CI: Confidence interval; IAs: Intermediate alleles; DLB: Lewy body Dementia; MSA: Multiple system atrophy; PD: Parkinson's disease; PDD: Parkinson's disease dementia.*

Table 5. Clinical and demographic data of patients carrying *HTT* IAs.

MSA with neuropathological diagnosis								
ID	MSA clinical subtype	SND	OPCA	Gender	Age at onset	Age at death/las visit	*HTT* CAG allele 1	*HTT* CAG allele 2
MSA-1	MSA-C	I-II	III	Male	53	62	23	30
MSA-2	MSA-P	III	II	Female	Unknown	69	18	32
MSA-3	MSA-P	II	I	Female	58	65	17	30
MSA-4	MSA-P	II-III	III	Female	53	68	18	32
MSA-5	MSA-UD	II-III	II-III	Female	46	55	16	27
MSA with clinical diagnosis								
MSA-6	MSA-P		Possible	Female	65	72	18	29
MSA-7	MSA-P		Posible	Male	63	67	29	29

MSA-8	MSA-P		Probable	Male	69	75	20	29
MSA-9	MSA-C		Unknown	Female	61	65	15	34
MSA-10	MSA-P		Unknown	Female	66	71	17	28

SND= striatonigral degeneration; OPCA= olivopontocerebellar atrophy; MSA-C=Multiple system atrophy- cerebellar; MSA-P= Multiple system atrophy-parkinsonian type; BG=basal ganglia; UD= undefined



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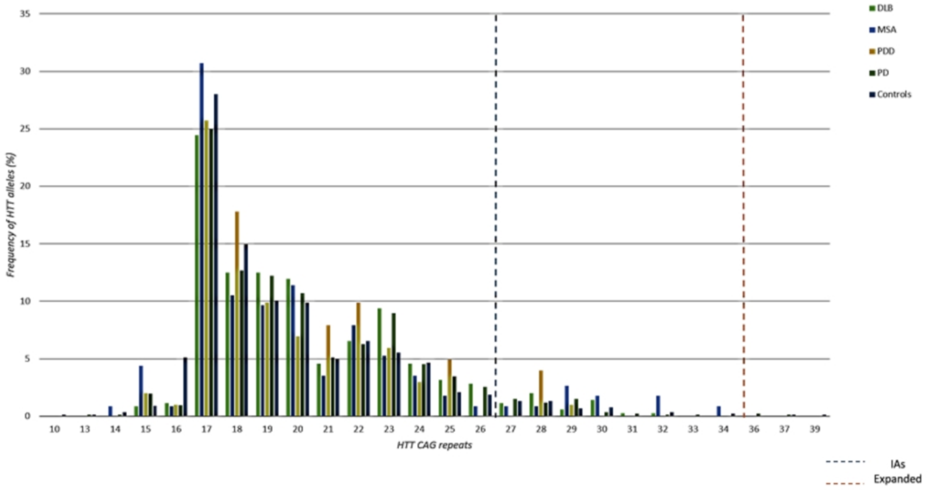

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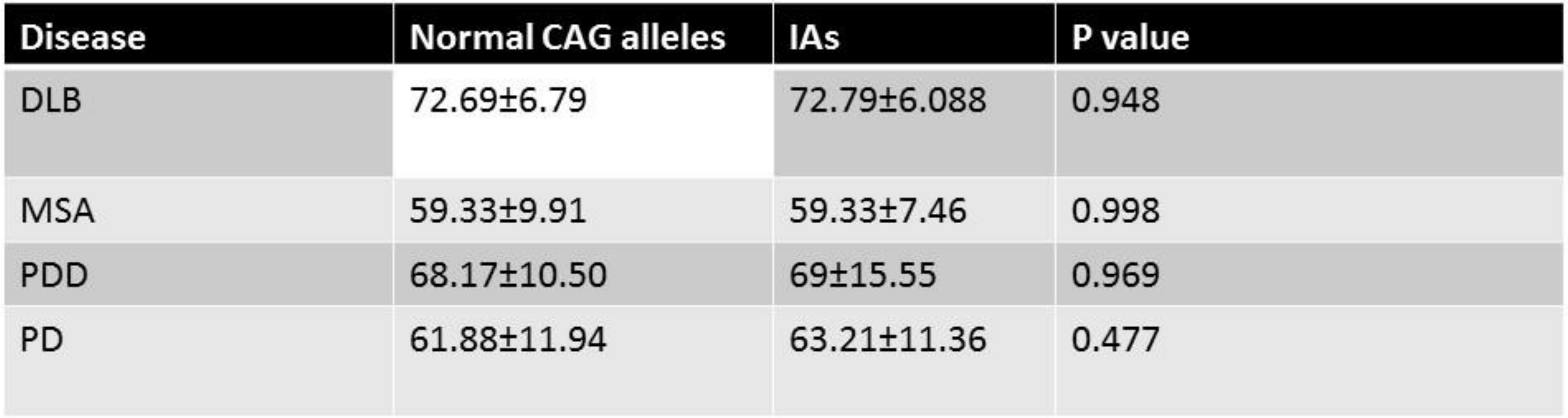
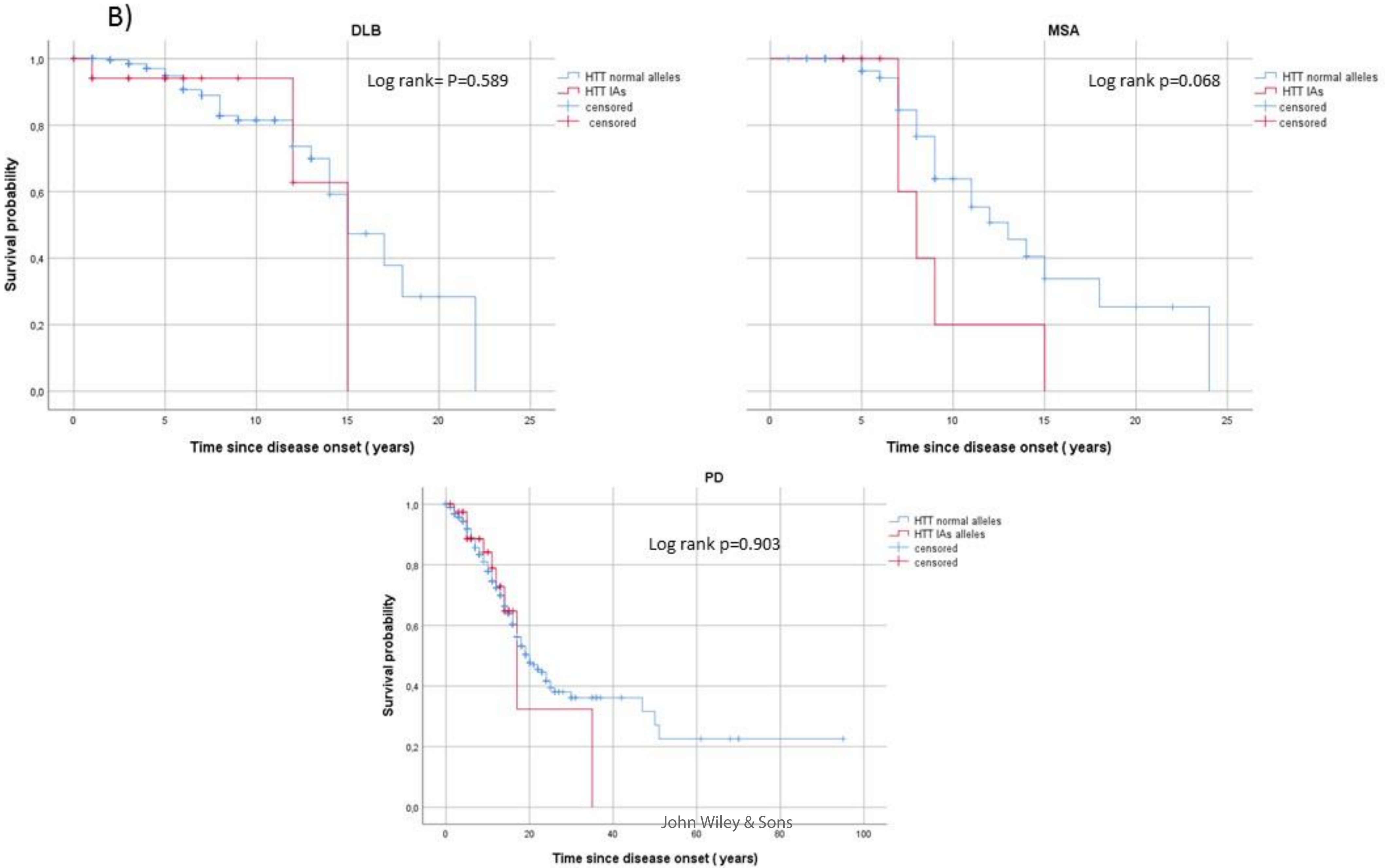
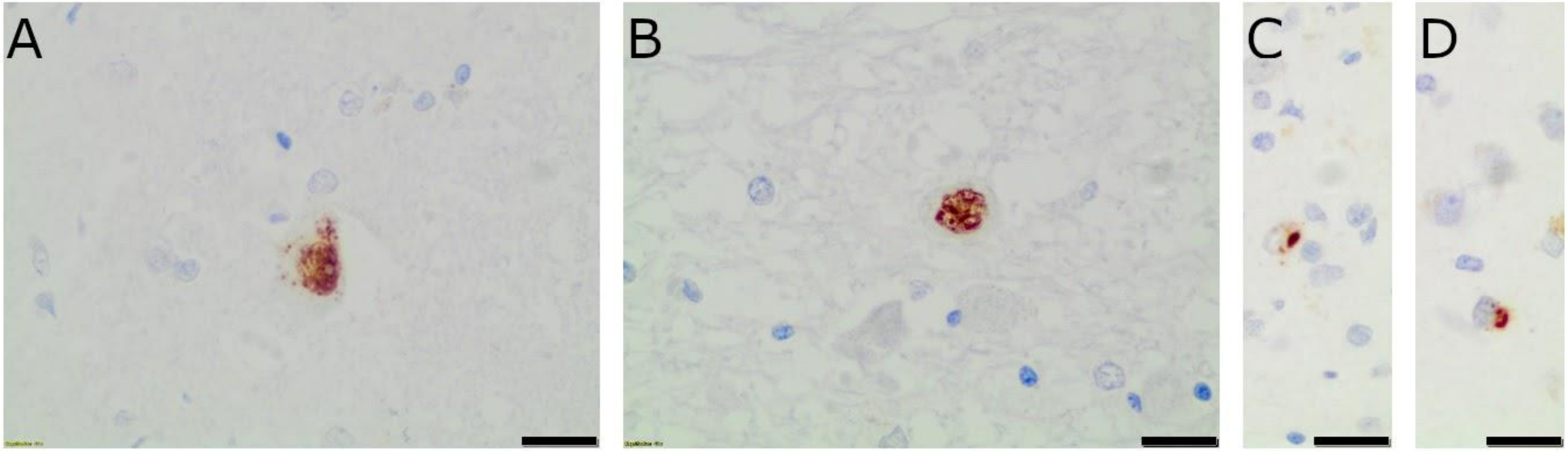
A)



Disease	Normal CAG alleles	IAs	P value
DLB	72.69±6.79	72.79±6.088	0.948
MSA	59.33±9.91	59.33±7.46	0.998
PDD	68.17±10.50	69±15.55	0.969
PD	61.88±11.94	63.21±11.36	0.477


B)
DLB
Log rank= P=0.589
HTT normal alleles
HTT IAs
censored
censored
Survival probability
1,0
0,8
0,6
0,4
0,2
0,0
0
5
10
15
20
25
Time since disease onset (years)
MSA
Log rank p=0.068
HTT normal alleles
HTT IAs
censored
censored
Time since disease onset (years)
PD
Log rank p=0.903
HTT normal alleles
HTT IAs alleles
censored
censored
Survival probability
0
20
40
60
80
100
Time since disease onset (years)


A
B
C
D

Supplementary Table 1. *Comparison of the IAs distribution between genders in the different groups.*

Neuropathology	**Male**	**Female**	**p-value; OR (IC-95%)**
DLB	6 (3.2)	14 (8.5)	**0.03774***; 2.82 (0.99-9.18)
MSA	3 (5.9)	7 (11.1)	0.5079; 1.99 (0.42-12.57)
PDD	2 (3.0)	3 (4.8)	0.6709; 1.65 (0.18-20.34)
PD	23 (4.0)	19 (4.5)	0.7507; 1.14 (0.58-2.21)
Controls	15 (3.2)	24 (4.4)	0.3296; 1.41 (0.70-2.93)

** Significantly different among groups (p < .05). OR: Odds ratio; CI: Confidence interval. DLB: Lewy body Dementia; MSA: Multiple system atrophy; PD: Parkinson disease; PDD: Parkinson´ disease dementia.*

Supplementary Table 2- Neuropathological findings after Poli Q IHQ

	frontal cortex	*caudate*	*putamen*	*globus pallidus*	*nucleus basalis*	*pons with locus ceruleus*	*cerebellum and dentate nuclei*	*HTT CAG allele size (allele 1/allele 2)*
MSA-1	0	NA	NA	NA	NA	NA	NA	23/30
MSA-2	0	NA	NA	NA	NA	+	0	18/32
MSA-3	0	0	0	0	0	0	0	17/30
MSA-4	0	0	+	0	0	0	0	18/32
MSA-5	0	0	0	0	0	0	0	16/27
MSA-C1	0	0	0	0	0	0	0	16/17
MSA-C2	0	0	0	0	0	0	0	22/24

MSA: Multiple system atrophy; NA: Not Applicable/not available.

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HTT Intermediate alleles' (IAs) frequency is increased in Multiple system atrophy. Two MSA IAs carriers showed isolated polyQ inclusions in pons and basal nuclei, which are two critical structures in the neurodegeneration of MSA. Our results pointed to a link between *HTT* CAG repeats with other non -HD brain pathology.