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HUNTINGTON'S DISEASE: GENETIC MODIFIERS OF AGE AT ONSET AND PATHOLOGICAL BIOMARKERS

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Index

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease, clinically characterized by movement disorders, cognitive deficits, and psychiatric problems. Although George Huntington first described this condition in 1872 (Huntington, 1872), the identification of the disease-gene and the characterization of the genotype/phenotype relationship belong only to the last three decades (Walker, 2007).

HD is one of the most common among the rare neurogenetic disorders but epidemiological data differ from one ethnicity to another. HD is more frequent in Caucasian than in Asian and African populations (Rawlins, 2016). Global prevalence is reported as 10.85 affected individuals per 100 000 in Italy (Squitieri, 2015). Highest incidences can be found in areas where a founder effect is recognised, as Tasmania (Pridnore, 1990) or the regions around Lake Maracaibo in Venezuela (Young, 1986).

The gene responsible for HD is *IT15* and it is located on the short arm of chromosome 4 (The HD Collaborative Research Group, 1993). The gene encodes the protein huntingtin (HTT), expressed in all human cells with highest concentration in Central Nervous System (DiFiglia, 1995). Mechanisms of action of this protein are poorly understood. HTT seems to indirectly regulate the transcriptional processes and participate in long- and short-range axonal transport. These functions could be essential for embryonic development in animals: homozygous knockout mice for *IT15* exhibit embryonal lethality by day 8.5 (Nasir, 1995). HTT has been supposed to regulate the production and vesicular trafficking of the brain-derived neurotrophic factor (BDNF) (Zuccato, 2001; Gauthier, 2004).

An increased cellular death rate can also be observed in HTT knockout animals and a possible antiapoptotic role has been hypothesized. This protein could bind and prevent the formation of the pro-apoptotic Hip1-HIPPI (Hip1 protein interactor) complex or directly block the activation caspase 3 and 9 (Gervais, 2002; Rigamonti, 2001).

The gene *IT15* contains a variable number of CAG (Cytosine-Adenine-Guanine) trinucleotides within the exon 1. Healthy individuals have fewer than 36 CAG repeats and most commonly about 15–25 repeats. CAG trinucleotides result in a polyglutamine tract (poliQ) within the N-terminus of the HTT protein. When their number exceeds the threshold of 35, the protein loses its physiologic structure and function, causing the clinical manifestations of HD. CAG repeats that exceed the number of 28, show instability on replication and their size could increase during the meiosis, more in spermatogenesis than oogenesis (Duyao, 1993). A correlation exists between CAG repetitions, severity of neurodegenerative mechanisms, and the clinical features of HD (Rubinsztein, 1993). The higher is the number of CAG in the expanded allele, the longer is the pathological poliQ in the mutant HTT (mHTT), and the earlier is the age at onset (AO) and the faster the clinical course of the disease, due to a more severe diffuse neuronal impairment. According to data in larger cohorts of HD patients, the number of CAG repeats has been estimated to account for about 56%-70% of the variation in age of onset (Andrew, 1993; Snell, 1993). Meiotic instability and correlation genotype-phenotype explain the phenomenon of anticipation, typical for HD and all the trinucleotide expansion disorders (Spinocerebellar ataxias, Dentato-rubro-pallido-luysian atrophy, X-linked Spino-bulbar muscular atrophy): the AO becomes progressively earlier from a generation to the next (Ridley, 1988).

Pathological CAG expansion in *IT15* confers toxic functions to HTT. N-terminal fragments (containing the expanded poliQ), full-length mHTT monomers, oligomers, and large mHTT aggregates coexist in HD pathogenesis and disrupt multiple cellular pathways (Labbadia, 2013). MHTT shuttles into the nucleus where it affects the recruitment of RNA polymerase II and modifies gene expression through the binding of many transcription factors and cofactors, including CREB-binding protein (CBP), p300/CBP-associated factor (p/CAF), p53, SP1, TAFII130, and PQBO1 (Seredenina. 2012; Okazawa, 2003). MHTT associates with the outer membrane of mitochondria, impairing its functions: it reduces energy production and membrane potential, alters calcium homeostasis, enhances oxidative stress, and triggers the caspase-mediated apoptotic pathways (Mochel, 2011; Browne, 1997). MHTT dysregulates mitochondrial biogenesis and function through effects on *PGC-1a* gene expression, and impedes retrograde and anterograde mitochondrial trafficking along axons (Cui, 2006). Dysfunction of the ubiquitin–proteasome system (UPS) has been demonstrated in HD (Li, 2011; Orr, 2008). Aggregates of mHTT accumulate into the cytosol where they sequestrate the key UPS components and prevent delivery of misfolded proteins to the nuclear proteasome (Park, 2013). These inclusions can also reduce the levels of HSP70 and DNAJ chaperons, through a combination of sequestration and transcriptional dysregulation, disrupting the cellular protein homeostasis (Hay, 2004). MHTT dramatically impairs neurotransmitters release at presynaptic junctions by physically impeding axonal transport and by reducing the efficiency with which synapse-bound cargo can be loaded onto microtubules (Morfini, 2009).

Although the presence of mHTT is widely deleterious for the Central Nervous System, medium-sized spiny neurons (MSNs) of the striatum exhibit enhanced vulnerability (Ferrante, 1991). Possible explanations consider both susceptibility to

loss of BDNF neurotrophic support from cortical-striatal fibres and, conversely, sensitivity to excitotoxicity arising from cortical glutamatergic projections (Okamoto, 2009; Zuccato, 2009). At a later stage, pathological processes also affects other brain regions, including cerebral cortex (layers III, V, and VI), hippocampus, thalamus, globus pallidus, subthalamic nucleus, substantia nigra, white matter, and cerebellum (Vonsattel, 2008).

From a histopathological point of view, even before the symptomatic manifestations of HD, immunohistochemical analyses demonstrate the presence of aggregates of polyubiquitinated mHTT that can form neuronal intranuclear or cytoplasmic and neuropil extranuclear inclusions both in the striatum and throughout the cortex (Gutekunst, 1999). In the last stages, cellular death processes and reparative gliosis phenomena can be observed.

The onset of HD is conventionally defined as motor onset and the typical motor disorder is represented by choreic movements. 70% of HD patients are believed to have chorea at onset or during the clinical course of disease (Gudesblatt, 2011). As defined by Sanger, chorea is an ongoing random-appearing sequence of one or more discrete involuntary movements or movement fragments. Movements appear random due to variability in timing, duration, or direction (Sanger, 2010). At HD onset, choreic movements appear subtle, mild, and limited to face and/or distal extremities. They are usually worsened by anxiety and psychological stress and subsides during sleep. HD patients attempt to disguise chorea by incorporating it into a purposeful activity and this causes a condition of "restlessness"; as the disease worsens, chorea may become more proximal and involve the trunk, impairing gross motor coordination skills, gait and balance. Another pathognomonic finding in HD is the motor impersistence, which is the inability to maintain a voluntary muscle

contraction at a consistent level (Gordon, 2000). This difficulty leads to changes in position and sometimes compensatory repositioning, contributing to the HD patients' overactivity. Motor impersistence is independent of chorea and is linearly progressive (Reilmann, 2001). Oculomotor abnormalities are well recognised in HD and can sometimes anticipate the choreic disorder. Both saccades and smooth pursuit movements may be abnormal: the first can be slow or reduced in latency and amplitude, the second are jerky or incomplete more frequently (Lasker, 1997). Dystonic postures and parkinsonian manifestations (mainly bradykinesia and rigidity) may coexist with chorea and participate to the motor decline during the disease progression. Their prevalence is greater in the juvenile forms of HD (Westphal variant of HD) or in the latest stage of HD, when chorea becomes less apparent (Nance, 2001).

Psychiatric and behavioural problems combine with motor symptoms in a variable percentage of about 33%-76% and their expression is very variable (van Duijn, 2007). Mood depression is the most frequently reported condition and its prevalence amounts to more than twice that found in general population. Depressive symptoms frequently appear precociously and diminish during the middle and later stages of the disease. Around 10% of HD patients report suicide attempt but depression is not always the cause (Paulsen, 2005). Suicidality can be sometimes considered as expression of impulsive behaviour. In association or not with depression, HD patients may suffer from apathy, anxiety, and obsessive-compulsive behaviour. Irritability is another common sign and it often occurs in the earlier stages. Its severity varies enormously from serious disputes to physical aggression (van Duijn, 2007). Psychosis is more frequent in the latest stages and it is associated with a more relevant cognitive decline. Paranoid delirium and acoustic hallucination characterize the clinical picture.

Cognitive problems complete the clinical triad of HD. Degree of severity and time at onset differ from one patient to another. The first cognitive alterations typically involve the executive function. Mind flexibility, judgement capability, and planning skills are first compromised; long-term memory and language are initially spared (Snowden, 2017). In the latest stages of disease, a progression in subcortical dementia is often described (Brandt, 1886).

Diagnosis of HD is genetic and a pathological CAG expansion is researched in *IT15* gene. Genetic test can be required for patients with a clinical picture suggestive of HD (diagnostic test) or for healthy individuals with a positive familial history of HD (predictive test). Neuroimaging studies can strengthen the clinical suspicion. Routine MRI and CT show a loss of striatal volume and increased size of the frontal horns of the lateral ventricles in moderate-to-severe HD patients (Strober, 1984). F^{18}-FDG-PET could show a striatal and cortical reduction of radiotracer uptake already in the early stages of disease (Ciarmiello, 2006).

No therapy is currently available to delay the onset of symptoms or prevent the progression of the disease but symptomatic treatment may improve the quality of life and prevent complications. Choreic movements should be treated pharmacologically only if they become disabling to the patient and tetrabenazine, a dopamine-depleting agent, is the most commonly used drug for this purpose. Selective serotonin reuptake inhibitors (SSRIs), Serotonin–norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants, mood stabilizer drugs, and typical and atypical neuroleptics are variably chosen for the control of psychiatric and behavioural problems (Frank, 2014). No specific treatments exist for the cognitive deficits in HD. Experimental therapies, aimed to avoid the expression of the mHTT, are being tested in animal models and human trials.

Perceived motor, cognitive and psychiatric onset in Huntington's disease: a correlation analysis.

Clinical attention has been historically directed towards motor symptoms in HD although motor abnormalities represent only one aspect of the clinical triad of the disorder.

Today again HD onset is considered as motor onset, patients without motor symptoms are defined prodromal, and genetic test is considered predictive although a patient with a positive familial anamnesis for HD, shows behavioural problems or describes attentional deficits. In experimental trials, inclusion criteria exclusively consider the motor onset and primary end-points look at a change in motor status.

Only in the last decade, cognitive and psychiatric problem have received the proper attention, mainly due to the work of the PREDICT-HD study group. Now we know that cognitive and psychiatric symptoms are equally relevant to motor problems in HD and can compromise the quality of life of the patients and their caregivers. They sometime represent the more relevant problems to manage in HD clinical course.

Cognitive and psychiatric impairments may precede choreic movements or other movements disorders in HD by a decade.

Frontal functions are first impaired in HD and related to basal ganglia-thalamocortical circuits dysfunctions. HD patients early experiment difficulties in problem solving, events planning, and decision making processes. A condition of mild cognitive impairment, due to the impairment of these functions, is then

recognisable, presenting in up to half of premanifest persons close to motor diagnosis (Duff, 2010).

Before the onset of motor symptoms, cognitive alteration can progress and become a self-perceived problem, able to interfere in the functional levels (Paulsen, 2013).

Beglinger and her colleagues conducted one of the most detailed analyses involving predictors of patient-centered outcomes and reported that performance on the Stroop Interference Task was associated with functional loss for the ability to manage finances, drive safely, supervise children, volunteer, and grocery shop. The Symbol Digit Modalities Test was associated with inability to grocery shop and use public transportation (Beglinger, 2010).

Anxiety and depression often represent the first psychiatric problems in HD. Since the onset, changes can be appreciated in behaviour modalities and interaction with family members and friends. Increasing difficulty in managing emotions sometimes leads to aggressive outbursts and “catastrophic reactions” (Almqvist, 1999). Anxiety can manifest with psychosomatic symptoms, such as gastrointestinal problems or insomnia, which need symptomatic treatment. (Verny, 2007).

Apathy, irritability, and depression seem to worsen over time in the earliest stages of HD (Stage I and II), and only apathy continued to progress with disease progression into Stages II–IV (Craufurd, 2001). In another study, a cohort of 45 prodromal gene mutation carriers was studied over an interval of 3.7 years: all the psychiatric problems increased but the greatest changes were noted in Irritability and Hostility (Kirkwood, 2002). More recently, Tabrizi and colleagues followed

120 prodromal Huntington disease gene mutation carriers and reported worsening apathy over 36 months (Tabrizi, 2013).

Follow-up of the psychiatric problems in HD can be biased the awareness of the symptoms. The PREDICT-HD study group evidenced that companions of HD mutation carriers also report greater psychiatric symptoms over time compared to affected individuals. It is important to note that in HD mutation carriers who do not yet have motor diagnosis, decreased awareness of both motor (McCusker, 2010) and psychiatric (Duff, 2010) symptoms have been documented.

A correlation between CAG repeats number and motor onset is well-known in HD; a little is reported about the correlation between CAG repeats in pathological *IT15* allele and non-motor onset or if motor onset is associated to the occurrence of cognitive deficit or psychiatric problems. Our study is aimed to investigate these aspects in 188 patients with HD.

Patients and methods

One hundred and eighty-eight patients (96 M, 92 F) with HD have been enrolled in this study. Only HD patients with available data on age, sex, age at symptom onset, mutant CAG repeat size were included in this study.

In the examined cohort, the expanded trinucleotide repeats ranged from 40 to 63 with a mean (± SD) of 44.95 ± 3.63 CAGs.

Age at symptoms in HD cohorts was defined as the age at which the first signs of HD appeared, distinguish between motor disturbances, psychiatric problems, and cognitive decline. The time at onset of motor (AMO), cognitive (ACO), and

psychiatric problems (APO) has been anamnestically deduced, interweaving the patients and their caregivers. For the psychiatric problems, time at onset was investigated about the occurrence of Depression, Irritability, Violent or Aggressive Behaviour, Apathy, Perseverative/Obsessive Behaviour, and Psychotic symptoms.

Statistical Analysis

As the relation between AO and mutant CAG repeat size is known to be exponential (Langbhen, 2004), we used the logarithmic transform of AO (AMO, ACO, APO) as dependent variable. The sizes of the mutant CAG repeats in *IT15* gene has been used as predictor variables.

Univariate linear regression analyses will be first performed to determine the effect of the expanded HTT allele on AMO, ACO, and APO. A correlation analysis was conducted to study the relationship between the modalities of AO.

Results

Onset data in HD patients are summarized in Table 1.1. For 93.08%, 68.08%, and 92.08 of HD patients a motor, cognitive and psychiatric onset was respectively datable. For 30.85%, 3.72%, and 37.76% of HD patients the first symptoms were respectively only motor symptoms, cognitive deficits, or psychiatric problems. In 26.67% of our cohort, there was a combination of these symptoms at onset.

A regression equation used to model AO of HD patient is $LAO = \alpha + \beta X$, where X is the expanded CAG repeat length and LAO is the natural log of AO.

First, we plotted the *IT15* pathological CAG repeat length for the longer HD ($CAGs^{HD}$) allele against the LAO for each individual in our cohort. For the logarithmic AMO, regression equation was LAO = 2.80 – 0.03 $CAGs^{HD}$, Pearson's r was -0.80, and r^2 value was 0.65 ($p < 0.01$). For the logarithmic ACO, regression equation was LAO = 155.51 – 2.43 $CAGs^{HD}$, Pearson's r was -0.73, and r^2 value was 0.54 ($p < 0.01$). For the logarithmic APO, regression equation was LAO = 2.56 – 0.02 $CAGs^{HDl}$, Pearson's r was -0.55, and r^2 value was 0, 30 ($p < 0.01$).

When the psychiatric onset symptoms were singularly studied, using as dependent variable their logarithmic time at onset and CAG repeats expansion as predictor variable, statistically significant ($p < 0.01$) regression models were obtained for all the psychiatric onset modalities with the exception of Psychiatric Symptoms. Regression equation, Pearson's r and r^2 value are summarised in Table 1.2.

In the correlation analysis among modalities of onset, all the correlation were statistically significant. A higher rho value was obtain between motor and cognitive onsets ($\rho = 0.92$); lower was the rho values between motor and psychiatric onsets ($\rho = 0.74$) and between cognitive and psychiatric ($\rho = 0.71$). When the single psychiatric domains were considered, the higher correlations with motor and cognitive onsets concerned Apathy and Perseverative/Obsessive Behaviour. When the single psychiatric domains were compared among them, the stronger correlations were those among Depression, Irritability and Violent or Aggressive Behaviour. Correlation analyses results are reported in Table 1.3.

Discussion

Our study aims to investigate the dependence of motor, cognitive and psychiatric onsets from CAG repeats length in *IT15* pathological allele and to study the correlation among these several modalities of clinical debut. The study has analysed these data in a large cohort of Italian HD patients. We have used anamnestic data, referring to the onset perceived from patients and their companions. Our study doesn't report alterations in the subclinical phase and we think that our results may be closer to the daily life of HD patients. This type of source data could represent a potential study bias. At same time, the problem of patients' awareness could interfere with the results. To limit this bias and to have a more accuracy of data, we have required the presence of the companion during the interview.

In our patients, psychiatric and motor problems often represent the first symptoms, alone or in combination. The isolate cognitive onset is very rare. Association between onset modalities has been demonstrated through a correlation study. The stronger correlation is that between motor and cognitive onset: the earlier is the motor onset, the earlier are the cognitive problems. This association can be related to mechanism that are involved both in motor symptoms occurrence and cognitive problems debut: the basal ganglia-thalamocortical circuits dysfunctions. Lower but always consistent correlations are present between psychiatric onset and motor onset and between cognitive onset and psychiatric onset. However, when a subanalysis was performed in relation to the different psychiatric symptoms, we have found that Perseverative/Obsessive Behaviour and Apathy have considerable positive correlation levels with motor and cognitive onsets, due to the same

mechanisms, the alteration of the connections between basal ganglia and prefrontal cortex (Huey ED, 2008; Lewy 2006).

Our study confirm the strength correlation between CAG repeats length and motor onset. In this study CAG onset accounts for the 65% of variability in motor onset. Our results confirm data in literature. We evidenced that also cognitive onset can depend from CAG repeats in *IT15* pathological allele: the length accounts for the 54% of variability in ACO. Onset in cognitive changes has been significantly ($r = 0.57$) associated to CAG repeats in the past literature (Andrew, 1993). The lower dependence degree between CAG repeats length and AO is that for psychiatric onset. CAG repeats number accounts only for the 30% in variability of APO. Also in this case we confirm data reported in other studies. Andrew et al. first evidence a statistically significant correlation ($r = 0.51$) between CAG and psychiatric onset (Andrew, 1993) and later studies found that the 31%-50% of variability in psychiatric onset is explained by CAG length (Squitieri, 2001, Vassos, 2008). The low dependence levels that we find, could be related to the bias that we have already mentioned. Another problems is to differentiate psychiatric problems due to structural and functional alterations from those related to an endoreattive aetiology. However we have found that CAG expansion explains a different level of variability in onset for every different psychiatric domain examined: it accounts for the 60% and 50% in variability in onset for Apathy and Depression respectively.

Our study demonstrates the relevance of cognitive and psychiatric onsets, due to their frequency in HD clinical course and correlation with motor onset. To recognise psychiatric or cognitive problems as HD onsets could favour the patients to an early access to the diagnostic course and to experimental drugs. Variation in

cognitive and psychiatric variables has to represent recognised markers of pathology, similarly to motor factors.

The study demonstrates that CAG length represents a relevant but not the only factor responsible for cognitive and psychiatric onsets variability, as reported for motor onset. Modifier factors which are recognise to modulate the motor onset, should be also investigated for the debut of cognitive and psychiatric problems.

Tables

	HD patients
	(96 M, 92 F)
Expanded IT 15 gene (CAG n° mean ± SD)	44,95 ± 3,63
Motor onset (years mean ± SD)	44,02 ± 10,83
Cognitive onset (years mean ± SD)	46,52 ± 11,69
Psychiatric onset (years mean ± SD)	41,43 ± 12,26
Onset of Depression (years mean ± SD)	43,42 ± 11,75
Onset of Irritability (years mean ± SD)	44,52 ± 12,10
Onset of Violent or Aggressive Behaviour (years mean ± SD)	43,87 ± 11,26
Onset of Apathy (years mean ± SD)	47,23 ± 11,96
Onset of Perseverative or Obsessive Behaviour (years mean ± SD)	45,06 ± 12,78
Onset of Psychotic symptoms (years mean ± SD)	47,53 ± 12,77

Table 1.1. Demographic and genetic data.

Onset	***Sample n°***	***α***	***β***	***p***	***R square***
MOTOR ONSET	170	0,03	2,80	< 0,01	0,65
COGNITIVE ONSET	125	-2,43	155,51	< 0,01	0,54
PSYCHIATRIC ONSET	163	-0,02	2,56	< 0,01	0,30
Depression	139	-0,02	2,68	< 0,01	0,49
Irritability	136	-0,02	2,55	< 0,01	0,34
Violent or Aggressive Behaviour	82	-0,02	2,35	< 0,01	0,23
Apathy	94	-0,02	2,75	< 0,01	0,60
Perseverative or Obsessive Behaviour	83	-0,02	2,66	< 0,01	0,43
Psychotic symptoms	29	-0,10	2,26	0,10	0,06

Table 1.2. Regression equation.

Results of fitting linear regression ln(AO) = A + B(CAG)n. p value refers to the significance of the regression parameter (B) indexing the effect of repeat length. R square is the coefficient of determination and represents the proportion of the variance in the dependent variable that is predictable from the independent variable.*

	MOTOR ONSET	***COGNITIVE ONSET***	***PSYCHIATRIC ONSET***	*Depression*	*Irritability*	*Violent or Aggressive Behaviour*	*Apathy*	*Perseverative or Obsessive Behaviour*	*Psychotic symptoms*
MOTOR ONSET	-	0,92**	0,74**	0,80**	0,80**	0,71**	0,84**	0,84**	0,78**
COGNITIVE ONSET	0,92**	-	0,72**	0,78**	0,78**	0,66**	0,86**	0,91**	0,74**
PSYCHIATRIC ONSET	0,74**	0,71**	-	-	-	-	-	-	-
Depression	0,80**	0,78**	-		0,99**	0,98**	0,86**	0,76**	0,72**
Irritability	0,80**	0,78**	-	0,99**		0,98**	0,86**	0,76**	0,72**
Violent or Aggressive Behaviour	0,71**	0,66**	-	0,86**	0,98**		0,76**	0,69**	0,74**
Apathy	0,84**	0,86**	-	0,86**	0,86**	0,76**		0,81**	0,74**
Perseverative or Obsessive Behaviour	0,84**	0,91**	-	0,76**	0,76**	0,69**	0,81**		0,56*
Psychotic symptoms	0,78**	0,74**	-	0,72**	0,72**	0,74**	0,56**	0,67*	

Table 1.3. Correlation analysis among the different onset modalities.

Within the cells the Pearson correlation r coefficients are reported.

*** p < 0.01; * p < 0.05*

Study of SCAs genes as disease modifier in Huntington's disease

As previously said, HD exhibits the typical phenomenon of genetic anticipation and the symptoms of the disease appear earlier and more severe in subsequent generations due to meiotic instability, which increases the CAG repeats number. However, the CAG repeat expansion accounts only for approximately 56%-70% of the variation in age at onset in the 40 to 55 CAG repeat range associated typically with adult onset (Andrew, 1993; Snell, 1993). It is therefore possible to imagine that modifier genetic variants that segregate independently from the primary mutation, may influence the AO, and probably also progression and severity of disease.

Differences in genetic factors, environmental conditions and epigenetic patterns between individuals contribute to disease susceptibility and phenotypic variability. The study of these factors is more difficult as their contribution is limited and their number is large.

For more than a decade, genetic modifiers in HD have been investigated, mostly utilizing the candidate gene approach. Several polymorphisms in various candidate genes have already been investigated as potential modifiers in HD patients. These studies have concentrated mainly on genes thought to be functionally relevant to the disease or to interact functionally with elongated HTT (Arning, 2012).

The CAG repeat in non-expanded *IT15* allele has been studied as possible genetic modifier factor in a cohort 921 patients with HD. In patient with pathological CAG repeats in the lower range, the increasing size of the normal CAG correlates with more severe symptoms. In patient with CAG repeats expansion in

the higher range, the non-expanded CAG correlates with a less severe clinical condition (Aziz 2009).

Several genetic modifier factors have been researched analysing the glutamate-mediated excitotoxicity. Altered NMDA receptor function and increased neuronal sensitivity to excitotoxicity are candidate mechanisms for selective neuronal dysfunction and degeneration in HD. The TAA repeat polymorphism in the 3' untranslated region of *GRIK2*, which encodes the GluR6 subunit of the kainite receptor, is associated with earlier AO in HD. Single Nucleotide Polymorphisms (SNPs) in GRIN1 (Glutamate Ionotropic Receptor NMDA Type Subunit 1), GRIN2A (Glutamate Ionotropic Receptor NMDA Type Subunit 2A), and GRIN2B (Glutamate Ionotropic Receptor NMDA Type Subunit 2B) have been widely studied with contrasting results. To date, none of them has been functionally validated (Saft, 2011).

Among the HTT-interacting proteins, *TCERG1* and *HAP1* have been studied as possible modifier factors. The imperfect $(Gln\text{-}Ala)_n$ repeat of the gene *TCERG1*, a transcriptional factor, has been screened in HD patients and the longest allele, which comprises 313 bp, has been associated to a slightly earlier AO (Holbert, 2001). The polymorphism T441M in *HAP1* has been considered protective because homozygous patient developed first symptoms 8 years later as compared with other patients (Wu, 2009).

PPARγ coactivator 1α (PGC-1α) is a transcriptional coactivator that controls mitochondrial biogenesis and respiration. This protein has been studied as modifier gene because its functions are impaired due to the binding with HTT. In a large cohort of 854 European HD patients, the SNP s2970870 is associated to an earlier

onset in homozygous state while the polymorphism rs7665116 is responsible for a delay in onset (Che, 2011).

Many other genes are candidates as modifier genetic factors and their association with HD requires further validations. They are implicate in the stress response and apoptosis, BDNF pathway, autophagy, and DNA repair mechanisms.

In our study, we have decided to study seven SCAs genes (*ATXN1, ATXN2, ATXN3, CACNA1A*, *ATXN7, PPP2R2B,* and *TBP*) as modifying genes in HD due to genetic, pathological, and clinical similarities between HD and SCAs.

Patients and methods

Study Design

Fifty HD patients (32 M – 18 F) and 100 healthy controls (HC) (47 M – 53 F) have been enrolled in this study. Only HD patients with available data on age, sex, age at symptom onset, mutant CAG repeat size were included in this study; for the control group, exclusion criteria was a positive pathological anamnesis and/or a positive familial anamnesis for neurodegenerative diseases.

In the HD cohort, the expanded trinucleotide repeats ranged from 41 to 56 with a mean (± SD) of 44,42 ± 3,57 CAGs; CAG repeats number in the not expanded allele ranged from 9 to 25 with a mean (± SD) of 18,10 ± 3,22 CAGs

Age at symptoms in HD cohorts was defined as the age at which the first signs of HD appeared, distinguish between motor disturbances, psychiatric problems, and cognitive decline. Motor onset was reported in all the HD patients and mean age at motor onset (AMO) was 46,17 ± 12,52 years. Cognitive onset was reported in 36

patients (72% of the HD cohort) and mean age at cognitive onset (ACO) was 47,56 ± 12,14 years. Psychiatric onset was reported in 43 HD patients and mean age at psychiatric onset (APO) was 43,40 ± 15,50 years. Regarding psychiatric onset, 48,83% of HD patients had mood depression at onset, 23,25% had apathy, 39,53% had irritability, 18,60% had aggressiveness, and 6,97% had psychotic symptoms.

For every HD subject and HC, CAG repeats will be sized in the larger allele of *ATXN1, ATXN2, ATXN3, CACNA1A*, *ATXN7, PPP2R2B,* and *TBP* genes, according the following modalities.

Gene analysis

Genomic DNA was extracted from blood samples using a standard phenol-chloroform extraction.

We determined the presence and precise size of the triplet repeat expansion according to an established fluorescent repeat-primed PCR protocol [REF].

PCR was performed using GeneAmp 9700 thermal reaction cycler (Applied Biosystems, Foster City, CA) in a 20 µL PCR mixture containing, 1 µL of extracted DNA (15-50 ng/µl), 0.5 µM of each forward primer fluorescently labeled with FAM, HEX , NED or PET as shown on Table 2.1, 1 X FailSafe Premix K containing PCR buffer, dNTPs and MgCl2 (Epicentre, Madison, WI, USA), and 1.0 unit of Platinum Taq DNA polymerase (Invitrogen Corporation, CA, USA).

Amplification was performed with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 67°C for 1 minute and extension at 72°C for 3 minutes and a final 15 minute extension at 72°C.

One microliter of the generated PCR products was added to a mixture of 1.0 µL of LIZ 500 internal size standard (Celera diagnostics) and 9 µL of HiDi formamide (Invitrogen Corporation, CA, USA). The mixture was heated at 95ºC for 2 minutes and resolved by electrophoresis on an automated ABI Prism 3100 Genetic Analyzer using POP-6 (performance optimized polymer), on a 50 cm array (Applied Biosystems). Samples were electrokinetically injected at 15 kV for 10 seconds and electrophoresed at 15 kV for 5400 seconds at 60ºC under filter set D. Raw data were analyzed with GeneMarker 1.85 (SoftGenetics, State College, PA), programmed for automatic allele calling based on fragment sizes. Allele calls on GeneMarker 1.85 software were calculated using mean and 2 standard deviation of each allele type from different runs and by setting a minimum and maximum size range for each marker.

Statistical Analysis

As the relation between AO and mutant CAG repeat size is known to be exponential (Langbhen, 2004), we used the logarithmic transform of AO as dependent variable. The sizes of the mutant and normal CAG repeats in *IT15* gene and the sizes of CAG repeats in *ATXN1, ATXN2, ATXN3, CACNA1A, ATXN7, PPP2R2B,* and *TBP* genes were used as predictor variables.

Univariate linear regression analyses has been first performed to determine the effect of the expanded HTT allele and the normal HTT allele on AMO, ACO, and APO. Then, effects of the normal CAG repeats in seven SCA genes on AMO, ACO, and APO have been studied using linear regression analysis. Multiple regression analyses have been performed to study the additional effects on AO of SCAs genes, whose linear regression models have been found statistically significant.

As assessed by the Shapiro-Wilk test, CAG repeats in SCA genes did not meet the assumption for normality both in HD patients and in HC group. Accordingly, the Mann-Whitney U test was used to compare CAG repeats in *ATXN1, ATXN2, ATXN3, CACNA1A*, *ATXN7, PPP2R2B,* and *TBP* between HD patients and HC.

Results

Genetic data in HD patients and HC are summarized in Table 2.2.

A regression equation used to model AO of HD patient is LAO = α + βX, where X is the expanded CAG repeat length and LAO is the natural log of AO.

First, we plotted the *IT15* pathological CAG repeat length for the longer HD ($CAGs^{HDl}$) allele against the LAO for each individual in our cohort. For the logarithmic AMO, regression equation was LAO = 2.85 – 0.02 $CAGs^{HDl}$, Pearson's r was -0.79, and r^2 value was 0.62 ($p < 0.01$). For the logarithmic ACO, regression equation was LAO = 2.60 – 0.02 $CAGs^{HDl}$, Pearson's r was -0.6, and r^2 value was 0.60 ($p < 0.01$). For the logarithmic APO, regression equation was LAO = 2.50 – 0.02 $CAGs^{HDl}$, Pearson's r was -0.42, and r^2 value was 0,18 ($p < 0.01$).

Second, we plotted the *IT15* normal CAG repeat length for the shorter HD ($CAGs^{HDs}$) allele against the LAO for each individual in our cohort. For the motor onset as well as for onset, regression model was not statistically significant. For the logarithmic ACO, regression equation was LAO = 2.60 – 0.02 $CAGs^{HDl}$, Pearson's r was -0.6, and r^2 value was 0.60 ($p < 0.01$).

When the logarithmic AMO was considered, the simple linear regression analysis provided a statistically significant model only for the larger CAG expansion of *ATXN2* and *CACNA1A* genes. For *ATXN2,* the regression analysis was

LAO = 1.49 + 0.01 $CAGs^{ATNN2}$, Pearson's r value was 0.33, and r^2 value was 0.08. For *CACNA1A,* the regression analysis was LAO = 1.10 + 0.03 $CAGs^{CACNA1A}$, Pearson's r value was 0.34, and r^2 value was 0.12. When the logarithmic ACO was considered in association to SCAs genes, simple linear regression models were not statistically significant. For the logarithmic APO, the only statistically significant simple regression linear model was that for the larger CAG expansion of *ATXN2* gene. Regression equation was LAO = 1.37 + 0.02 $CAGs^{ATNN2}$, Pearson's r value was 0.35 and r^2 value was 0.12.

Simple linear regression equations for *IT15* and SCA genes are summarized in Table 2.3.

When a multiple regression model was built using the CAGs of *IT15* and *ATXN2* genes as predictor variables and the logarithmic AMO as dependent variable, the equation model was LAO = 2.62 - 0.02 $CAGs^{HDl}$ + 0.01 $CAGs^{ATXN2}$ and the adjusted r^2 was 0.55. When the same predictor variables were used to obtain a multiple regression model to study the logarithmic APO, the regression equation was LAO = 2.02 – 0.01 $CAGs^{HDl}$ + 0.01 $CAGs^{ATXN2}$ and the adjusted r^2 was 0.12.

In both cases, the two variables ($CAGs^{HDl}$ and $CAGs^{ATXN2}$) do not add statistically significance to the prediction, $p < .05$.

A multiple regression model was created to explain the logarithmic AMO starting from CAGs repeats of larger *IT15* and *CACNA1A* genes. The regression equation was LAO = 2.48 – 0.02 $CAGs^{HDl}$ + 0.02 $CAGs^{CACNA1A}$ and the adjusted r^2 was 0.64. In this case, the two variables ($CAGs^{HDl}$ and $CAGs^{CACNA1A}$) add statistically significance to the prediction, $p < .05$.

Statistical analysis demonstrated significant differences between HD patients and HC in CAG repeats number in *ATXN1, CACNA1A*, *ATXN7,* and *PPP2R2B* genes. A statistically significant increased number of CAG repeats in *CACNA1A* has been found in HD patients when compared to HD; contrarily, a statistically significant larger number in CAG repeats in *ATXN1, ATXN7,* and *PPP2R2B* has been found in HC compared to HD patients (Table 2.2).

Discussion

Disease onset is traditionally considered as motor onset in HD. Expanded CAG repeats account for more than 60% of AMO in HD; regression analysis in our cohort confirms this data. ACO and APO have not sufficiently studied in HD until today.

Several studies have investigated the remaining variability in AMO studying different genetic and environmental factors. At the current time, genes encoding for proteins which interact with HTT or modulate the same pathways, represent the most promising factors. Also the DNA repair genes seem to have a relevant role in modifying the AMO.

In this study we first investigate the possible role of SCA genes as modifier in AMO, ACO, and APO of HD patients. CAG repeats of normal alleles have been already considered when the non-expanded *IT15* allele has been examined. These studies did not show any correlation with AMO and our regression analysis confirm this evidence; only the stratification of HD patients on the basis of the pathological CAG repeats number, made possible to obtain positive findings (Aziz 2009).We have chosen to study the seven SCA genes for several reasons. HD and SCAs share the same genetic basis, the expansion of CAG repeats in a specific gene. The

pathological processes responsible for HD and SCAs could often involve the same brain structures. As in HD, striatal atrophy can be evidenced in SCA3 and SCA17 patients and dysfunction of the cortico-striato-thalamo-cortical loop is present also in SCA2, SCA6, and SCA8 (Park, 2015). On the other hand, cerebellum in HD shows a considerable atrophy, as well as a consistent loss of Purkinje cells and nerve cells of the fastigial, globose, emboliform and dentate nuclei (Rub, 2013). Clinically some SCA patients may present hyperkinetic movements, psychiatric problems and cognitive deficits; these SCAs, in particular SCA17, have been defined "HD-like syndromes" because they may be enter into differential diagnosis with HD (Martino, 2013). At the same time, HD patients can have cerebellar signs at onset or during the course of disease (Dong, 2013; Kageyama, 2003).

The mHTT accumulates into the cell and sequesters other proteins; among these proteins, we can find the non-expanded HTT. Due to a structural homology (presence of a poliQ trait) we can suppose that other proteins can be included into these intracellular aggregates, such as the wild-type proteins encoded by SCA genes. Anatomopathological studies hypothesize that polar zippers, formed by polyglutamine expansions, may recruit small polyglutamine tracts. In SCA7 brains, inclusion of pathological expanded ataxin-7 are immunostained by anti-ataxin-3, showing the presence of normal ataxin-3 within pathological aggregates of expanded ataxin-7 (Zander, 2001). This is the first evidence that expanded polyQ proteins could sequestrate not expanded poliQ proteins. In HD neurons, interaction between proteins containing poliQ tracts could occur and modulate the pathological potential of mHTT aggregates.

Lastly a pathological interaction between *IT15* and SCA genes has been already demonstrated. In a study aimed to investigate the modulation of the AO in SCAs

by CAG tracts in various genes, the authors found that the higher is the CAG repeats number (within normal range) of the longer *IT15* allele in SCA3 patients, the latter is the disease onset (Tezenas, 2014).

Our study doesn't find extensive correlations between CAG repeats in SCA genes and AMO, ACO, and APO of HD. The only exceptions are represented by *ATXN2* and *CACNA1* for AMO, and *ATXN2* for APO. When a multiple regression model was formulated and when the AMO was considered, a small additional effect (beyond that due to mutated *IT15*) was identified only for *CACNA1A*. CAG repeats in expanded *IT15* and larger *CACNA1A* alleles account for 64% of AMO in HD patients.

We also found differences in CAG repeats number in SCA genes between HD patients and HC. For *ATXN2*, *ATXN3*, and *TBP* there are no statistically significant differences between the two groups, whereas HD patients have a higher mean CAG number in *CACNA1A* and a lower mean CAG number in *ATXN1*, *ATXN7*, and *PPP2R2B* when compared to HC. It's interesting to note how *CACNA1A* is the only SCA gene which seems to modify AO jointly with *IT15*.

Statistically significant differences in CAG lengths of *ATXN1*, *CACNA1A*, *ATXN7*, and *PPP2R2B* genes between HD patients and HC may be expression of chromosomal instability. DNA chromosomal instability is typical in HD. Meiotic CAG instability is responsible for the phenomenon of anticipation. Interestingly, the expanded HD CAG repeat is also somatically unstable, undergoing progressive length increases over time: somatic instability is tissue-specific, with particularly high levels found in striatum and cortex and occurs in post-mitotic neurons. Global chromosomal instability has been demonstrated in HD: pluripotent stem cells (iPSC) were generated from primary dermal fibroblasts of HD patients and healthy

controls. Karyotype analysis revealed a significantly higher number of HD iPSC lines with genomic abnormalities when compared to control lines (Tidball AM, 2016).

CAG lengths appear increased in *CACNA1A* gene and reduced in *ATXN1*, *ATXN7*, and *PPP2R2B* genes; in *ATXN2*, *ATXN3*, and *TBP* genes, they don't differ from those observed in HC. This variability could reflect the heterogeneity of the instability mechanisms: they could involve some genes or spare others, producing an expansion or contraction of DNA tracts.

Although our study has analysed only a limited number of patients, we believe that these results could concur to the investigation of modifier factors in HD and the mechanisms of DNA instability, typical for this disorder. Further studies, conducted on larger HD patients' cohorts, are needed to confirm our data.

Tables

Primer name	*Forward primer sequence with fluorescent dye label on 5´*	*Reverse primer sequence*
SCA1	**/5HEX/**CTG AGC CAG ACG CCG GGA CAC AAG GCT GAG	AGC CCT GCT GAG GTG CTG CTG CTG CTG CTG
SCA2	/**5NED/**GAGCCGGTGTATGGGCCCC TCACCATGTCG	AGC CGC GGG CGG CGG **CTG CTG CTG CTG CTG**
SCA3	/**5PET/**CCA GTG ACT ACT TTG ATT CGT GAA ACA ATG	TCC TGA TAG GTC CCC **CTG CTG CTG CTG CTG**
SCA6	/5FAM/CAC GTG TCC TAT TCC CCT GTG ATC CGT AAG	CGG CCT GGC CAC CGC **CTG CTG CTG CTG CTG**
SCA7	**/5PET/**TGT TAC ATT GTA GGA GCG GAA AGA ATG TCG	CTG CGG AGG CGG CGG **CTG CTG CTG CTG CTG**
SCA17	/**5HEX/**CCT TAT GGC ACT GGA CTG ACC CCA CAG CCT	TGC CAC TGC CTG TTG CTG CTG CTG CTG CTG

Table 2.1. Primes used in PCR amplification.

	HD patients	*HC*	*p value*
***IT15* (HD)**	44.42 ± 3.57 / 18.10 ± 3.22	N.R.	
***ATXN1* (SCA1)**	35.72 ± 1.91	36.49 ± 1.60	< 0.01
***ATXN2* (SCA2)**	26.16 ± 1.28	26.28 ± 1.11	0.12
***ATXN3* (SCA3)**	26.83 ± 3.78	26.01 ± 5.23	0.353
***CACNA1A* (SCA6)**	17.18 ± 1.30	16.34 ± 1.19	< 0.01
***ATXN7* (SCA7)**	24.02 ± 2.23	25.62 ± 2.50	< 0.01
***PPP2R2B* (SCA12)**	17.38 ± 2.26	18.87 ± 2.85	< 0.01
***TBP* (SCA17)**	35.86 ± 1.20	35.73 ± 1.05	0.479

Table 2.2. Genetic data of HD patients and healthy controls (HC).

Data are reported as mean ± SD (standard deviation). For IT15 gene, the sizes of the smaller and the larger allele are expressed; for the other genes, only the size of the larger allele is reported. P value refers to Mann-Whitney test for comparisons HD group vs HC group.

		α	β	p	R square
Motor onset	*IT15 (*HD*) normal*	-6,44	28,78	0,10	0,06
	*IT15 (*HD*) expanded*	-0,03	2,85	< 0,01	0,62
	ATXN1 (SCA1)	1,78	-0,01	0,57	0,01
	ATXN2 (SCA2)	1,49	0,01	0,04	0,08
	ATXN3 (SCA3)	1,72	-0,01	0,56	0,01
	CACNA1A (SCA6)	1,10	0,03	0,02	0,12
	ATXN7 (SCA7)	1,26	0,02	0,34	0,02
	PPP2R2B (SCA12)	1,75	-0,01	0,48	0,01
	TBP (SCA17)	2,53	0,01	0,09	0,06

		α	β	p	R square
Cognitive onset	*IT15* (HD) *normal*	-8,63	32,35	0,01	0,08
	IT15 (HD) *expanded*	-0,02	2,60	< 0,01	0,36
	ATXN1 (SCA1)	1,84	-0,01	0,50	0,01
	ATXN2 (SCA2)	1,65	0,01	0,86	0,01
	ATXN3 (SCA3)	1,78	-0,01	0,38	0,02
	CACNA1A (SCA6)	1,31	0,02	0,12	0,06
	ATXN7 (SCA7)	0,95	0,03	0,12	0,07
	PPP2R2B (SCA12)	1,72	-0,01	0,73	0,01
	TBP (SCA17)	1,96	-0,01	0,61	0,01

		α	β	p	R square
Psychiatric onset	*IT15* (HD) *normal*	0,77	17,12	0,81	0,01
	IT15 (HD) expanded	-0,02	2,19	< 0,01	0,18
	ATXN1 (SCA1)	2,14	-0,03	0,09	0,06
	ATXN2 (SCA2)	1,37	0,02	0,04	0,12
	ATXN3 (SCA3)	1,68	-0,01	0,67	0,01
	CACNA1A (SCA6)	1,27	0,01	0,32	0,02
	ATXN7 (SCA7)	1,25	0,01	0,59	0,01
	PPP2R2B (SCA12)	1,52	0,01	0,66	0,01
	TBP (SCA17)	1,61	0,01	0,99	0,01

Table 2.3. Regression equations.

Results of linear regressions ln(AO) = A + B(CAG)n. p value refers to the significance of the regression parameter (B) indexing the effect of repeat length. R square is the coefficient of determination and represents the proportion of the variance in the dependent variable that is predictable from the independent variable.*

Peripheral markers of autophagy in Huntington's disease

Protein homeostasis is an indispensable requirement for neuronal survival and the synthesis of new proteins strictly follows the removal of damaged proteins. Ubiquitin–proteasome system (UPS) and autophagy represent the cellular mechanisms responsible for proteins degradation.

The UPS is the principal pathway for the clearance of short-lived, damaged, and misfolded proteins in the nucleus and cytoplasm (Dantuma, 2014). Ubiquitination and proteasomal degradation are the two separate and consecutive processes of UPS (Kleiger, 2014). An enzymatic cascade composed of ubiquitin activator, conjugase, and ligase catalyzes the covalent attachment of ubiquitin to a substrate protein. Multiple rounds of ubiquitination lead to the formation of a polyubiquitinated chain, which can function as a signal for degradation by the proteasome, a multisubunit enzyme complex composed of a 20S core particle and 19S regulatory particles, at one or both ends. The proteasome unfolds substrates and threads the polypeptide chains through the inner channel, where they are cleaved into short peptides (Bhattacharyya, 2014). Following their release from the barrel, peptides are rapidly processed into amino acids by cellular aminopeptidases and recycled (Reits, 2003).

Autophagy is a highly selective lysosomal mediated degradation process. Three major types of autophagy have been described in mammalian cells: macroautophagy, microautophagy and chaperone mediated autophagy (CMA). Macroautophagy is the only process that can mediate the degradation of larger substrates such as organelles, microbes and protein aggregates. (Lamark, 2012). It requires the isolation of substrates within a double membrane bound vesicles to

form the autophagosomes and, later, the fusion between autophagosomes and lysosome.

HD is characterized by cellular and nuclear accumulation of considerable amounts of mHTT. UPS cannot efficiently degrade polyQ-expanded proteins, as components of the UPS are frequently found in polyQ aggregates (Glickman, 2002). Failure of the UPS might lead to upregulation of autophagy via cross-talk between degradation pathways in the attempt by the cell to maintain normal proteostasis. One particular form of macroautophagy, called aggrephagy, is responsible for the selective elimination of accumulated and aggregated polyubiquitinated proteins (Yamamoto, 2011).

Aggrephagy involves a myriad of proteins, but three of them seem to be critical for the selective degradation of aggregates: microtubule associated protein 1 light chain 3 (LC3), p62, autophagy-linked FYVE domain protein (Alfy) (Clausen 2010). LC3, encoded by the gene *MAP1LC3*, is the mammalian homolog of Atg8 in yeast. It is essential for autophagosome biogenesis/maturation and it also functions as an adaptor protein for selective autophagy (Lee, 2016). The phosphatidyl-ethanolamine conjugated form of LC3 (LC3-II) is the activated form and is widely used as an autophagy marker. P62, also called optineurin and encoded by the gene *SQSTM1*, is a protein that binds both ubiquitinated cargos and LC3II, and functions as a signaling hub and an autophagy adaptor. P62 links ubiquitinated cargo with the nascent autophagosome allowing the maturation of the phagosome and subsequent fusion with lysosomes (Kasturagi, 2015). Alfy, encoded by the gene *WDFY3* is an autophagy scaffold protein that binds p62 and is required for the autophagic degradation of cytoplasmic ubiquitin-positive inclusions (Lee, 2016). After LC3,

Alfy and p62 binding, the autophagolysosome can be closed and the degradation of aggregates takes place (Filimonenko, 2010).

Although aggrephagy levels are preserved and even increased in HD, reports of cerebral biopsies from patients showed significant abnormalities in compartments of the vescicular-endocity pathway, noting an abnormal proliferation of lysosomes, HTT-positive autophagic vacuoles, and autophagolysosomes, as well as documenting disruption of the Golgi apparatus and disorganization of the endoplasmic reticulum (Tellez-Nagel, 1974). Therefore, the observed expansion of macroautophagy in HD cell is not accompanied by an expected increase in autophagy-mediated degradation. Cuervo et al. have explained this contradiction by demonstrating significant alteration in macroautophagy cargo recognition in HD cell cells, which leads to formation of empty autophagosomes (Martinez-Vicente, 2010).

Based on the presence of aggregated in polyQ diseases, we hypothesized an increase in peripheral autophagy markers. We designed a clinical study to measure the expression levels of *MAP1LC3*, *SQSTM1* and *WDFY3* in patients with HD, and to compare these levels with those found in healthy controls and patients suffering from SCA 2 (spino-cerebellar ataxia type 2), another polyglutamine repeat disorder. Expression levels were measured in peripheral blood mononuclear cells (PBMCs) as they represent an easily accessible and repeatable matrix for clinical use such as clinical trials or observational studies.

Patients and Methods

Study Design

This was a cross-sectional observational study to examine autophagy markers in patients with HD, SCA2 and in healthy individuals. Patients and controls were consecutively enrolled at our research center.

Inclusion criteria were a molecular diagnosis of HD or SCA2 for patients, and no evidence of known neurological and/or systemic disease for healthy controls.

Clinical Scales

The Scale for the Assessment and Rating of Ataxia (SARA) was used to assess the disability of patients with SCA2 (Schmitz-Hübsch, 2008) and the motor section of the Unified Huntington's Disease Rating Scale (UHDRS) for HD (Huntington Study group, 1996).

Gene analysis

Gene expression measurement was performed as reported elsewhere (Saccà, 2013). Briefly, peripheral blood mononuclear cells (PBMCs) were extracted from whole blood using Leucosep® tubes (Greiner bioone). Total mRNA from PBMCs was extracted with TRIzol® reagent (Thermofisher). Quality was checked with spectrophotometry and agarose-formaldehyde electrophoresis. One µg of total RNA was reversely transcribed (High Capacity RNA-to-cDNA, Thermofisher, USA), and 10% of cDNA product amplified using the TaqMan® Gene Expression

Master Mix. mRNA was quantified using Gene Expression Assays for MAP1LC3B, SQSTM1, WDFY3 (Thermofisher, ID n. Hs00917682_m1, Hs00177654_m1, Hs00698803_m1) and standardized by quantification of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Thermofisher, MGB probe) as a reference gene. We chose GAPDH as a reference gene after comparing the stability of GAPDH, Hypoxanthine Phosphoribosyltransferase 1 (HPRT1), and actin beta (ACTB) using normfinder software.25 Relative expression was calculated with the efficiency-calibrated model.26 The entire procedure followed the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.27

Statistical Analysis

Baseline variables analysis was conducted using the ANOVA, for three-group comparison, and the unpaired t-test for two-group comparison. We used the chi-square test to analyse gender distribution through groups. Normal distribution was tested with the Kolmogorov-Smirnov test. This showed a non-normal distribution for the relative expression of all tested genes, which was normalized through ln transformation. The difference in ln expression levels was analyzed with an Univariate General Linear Model that integrates a one-way ANOVA, phenotype as the only factor, and the Dunnett test as post-hoc. We used the Pearson's correlation coefficient for correlation analysis, and corrected for multiple comparisons using the Bonferroni correction.

P values of less than 0.05 were considered statistically significant. Statistical analysis was performed using SPSS version 23.0.0.1 running on macOS 10.11.6.

Results

We enrolled 20 patients with HD, 20 patient with SCA2 and 20 healthy controls. Demographics and clinical data are shown in Table 3.1. Disease duration and CAG (number of CAG repeats in the expanded allele) were significantly shorter for HD as compared to SCA2 patients. The remaining variables did not differ between groups.

MAP1LC3B ($p = 0.046$), SQSTM1 ($p = 0.001$), and WDFY3 ($p < 0.001$) significantly differed between groups (Fig. 3.1). HD patients showed higher expression levels of all three genes, whereas WDFY3 was the only overexpressed

gene in SCA2 patients (Table 3.2).

The following variables were used for the correlation analysis: age, disease duration, CAG2, clinical scale (UHDRS or SARA), Ln(expr_MAP1LC3B), Ln(expr_SQSTM1), and Ln(expr_WDFY3). Figure 3.2 shows the results for HD and controls. MAP1LC3B and SQSTM1 were positively correlated in all groups. SCA2 patients showed a positive correlation between MAP1LC3B and WDFY3, between WDFY3 and SQSTM1, between the SARA and both MAP1LC3B and SQSTM1.

Discussion

We report the first study measuring peripheral markers of autophagy in patients with HD and SCA2. Aggregate formation is a constant in all polyQ diseases, and due to the selectivity of aggrephagy in aggregate elimination, the value of peripheral markers as possible indicators of the process is evident.

MAP1LC3B, *SQSTM1*, and *WDFY3* were all increased in HD patients, suggesting a profound and intense induction in autophagy, aimed at counteracting the formation of protein aggregates. In contrast, SCA2 patients had a more modest modification with higher expression levels of WDFY3 alone.

This discrepancy may be due to the different pathological mechanisms of both diseases, as HD patients tend to form more aggregates than SCA2. Indeed, only cytoplasmic aggregates have a pathogenetic potential in SCA2, indicating a different nature of the aggregates themselves.

It was speculated that aggregates in SCA2 are made of Golgi fragments, and not of insoluble protein aggregates, as seen in HD. If this is true, in SCA2 the aggrephagic cascade, that is initiated by the contact of p62 with ubiquitinated proteins, is less activated as compared to HD.15 In addition, MAP1LC3B expression is strongly correlated with the number of autophagosomes,16 and since the latter are typically normal in SCA2 patients, and increased in HD, it is intuitive why MAP1LC3B levels were normal in SCA2.17

Recent evidence suggests that HTT is involved in the autophagic pathway in a specific way as it physically interacts with the autophagy cargo receptor p62 to facilitate its association with LC3 and with Lys-63-linked ubiquitin-modified substrates. The presence of empty autophagosomes in HD cells can be attributed to defective cargo recognition due to the presence of polyQ-htt in the inner part of the closing autophagosomes.18 According to our results, the increase in SQSTM1, WDFY3 and MAP1LC3B mRNA could be a compensatory mechanism in order to complete the autophagic process. Unfortunately this compensation is clearly not sufficient, as aggregates are not entirely removed and the disease still becomes manifest.

The absence of a clear correlation between clinical measures and markers of autophagy in HD patients, may prove that aggregate formation is independent of clinical parameters, or that autophagy is pushed to the maximum (i.e. it is saturated), so that correlation is lost. To support this, SCA2 patients, that have a lower level of aggregate formation, and limited increase in autophagy markers, show a correlation between disease severity and both MAP1LC3B and SQSTM1 expression. This is also supported by the higher disease duration in SCA2 patients that may have contributed to observed correlation.

Interestingly, MAP1LC3B and SQSTM1 expression were correlated throughout all phenotypes, including control individuals. This suggests that both genes may influence each other without influencing WDFY3, and that this mechanism takes place in physiological conditions as well as in the presence of aggregates.

A limitation of the present report is the cross-sectional nature of the study, that does not help in understanding the relationship between aggrephagy markers and polyQ diseases. Due to the progressive nature of both diseases, measurements in the same patients at different time-points of the disease could help better understand if disease progression and aggrephagy stimulation proceed in parallel, or if the two are independent. The same study in pre-symptomatic patients could prove that aggrephagy is over stimulated before disease onset, and if higher expression levels are a protective factor against phenoconversion to diagnosed HD.

In conclusion, our study shows that peripheral markers of autophagy are elevated in polyQ diseases, and that this is particularly evident in HD. These markers can be useful in future clinical trials aimed at modifying disease progression through different mechanisms, including stimulating autophagy.

Tables and Figures

	Controls	HD	SCA2	*p* value
Age	44.4 ± 8.8	53.2 ± 12.1	45.2 ± 13.0	0.052*/1.00**/0.090***
Gender (M/F)	9/11	8/12	13/7	0.247
Disease duration	–	6.0 ± 4.1	12.2 ± 10.1	*0.018*
CAG2	–	44.1 ± 2.6	43.1 ± 6.0	0.535
UHDRS	–	33.9 ± 21.9	–	–
SARA	–	–	20.3 ± 11.2	–

Table 3.1. Demographics and clinic data.

Italicized values correspond to significant p values

*HD Huntington's disease, SCA2 spinocerebellar disease type 2, UHDRS Unified Huntington's Disease Rating Scale, SARA Scale for the Rating and Assessment of Ataxia; CAG2 number of CAG repeats in the expanded allele. Age was preliminarily tested with a one-way ANOVA (p = 0.032) and Bonferroni's multiple comparisons test (*CNTRL vs HD; **CNTRL vs SCA2; ***HD vs SCA2). Differences in gender were tested with the chi-square test, differences in disease duration, CAG with an unpaired t test.*

Gene	Controls	HD	SCA2	HD vs controls (95% CI intervals)	Sig.	SCA2 vs controls (95% CI intervals)	*p* value
MAP1LC3B	0.99 (0.3)	1.42 (0.7)	1.08 (0.5)	0.43 (0.09; 0.77)	*0.048*	0.08 (− 0.25; 0.42)	0.978
SQSTM1	1.02 (0.2)	1.52 (0.5)	1.13 (0.6)	0.49 (0.20; 0.79)	*0.002*	0.11 (− 0.19; 0.40)	0.924
WDFY3	0.93 (03)	1.76 (0.6)	1.57 (0.5)	0.84 (0.53; 1.14)	*< 0.001*	0.65 (0.34; 0.96)	*< 0.001*

Table 3.2. Gene expression levels

Italicized values correspond to significant p values

HD Huntington's disease; SCA2 spinocerebellar ataxia type 2, CI confidence intervals. Gene expression levels are shown as relative expression, and standard deviation in parenthesis. Statistical analysis was performed on ln transformed values and analyzed with the Dunnett's post hoc test after passing the Univariate-GLM.

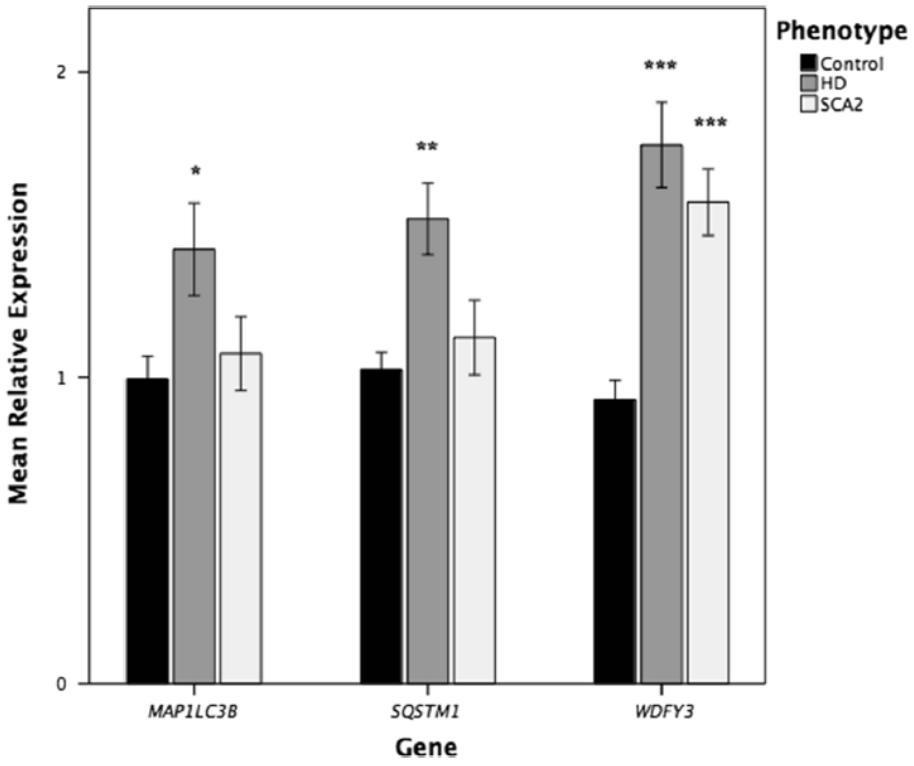

Fig. 3.1. Gene expression levels.

*Gene expression levels are shown as relative expression, whiskers show standard error mean. *p<0.05; **p<0.01; ***p < 0.001. Statistical analysis was performed on ln transformed values and analyzed with the Dunnett's post hoc test after passing the Univariate-GLM*

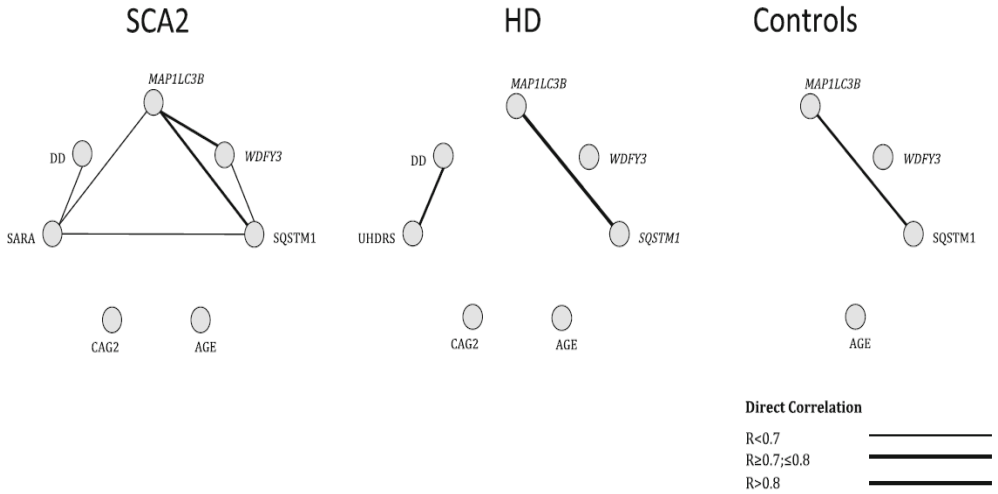

Fig. 3.2. Correlation analysis.

The figure shows correlations where $p < 0.05$ after Bonferroni correction for multiple comparisons. Correlation was performed using the Pearson's correlation coefficient and corrected for multiple comparisons using the Bonferroni correction. HD Huntington's disease, SCA2 spinocerebellar ataxia type 2, UHDRS Unified Huntington's Disease Rating Scale, SARA Scale for the Rating and Assessment of Ataxia, DD disease duration.

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