Distinct Brain Volume Changes Correlating with Clinical Stage, Disease Progression Rate, Mutation Size, and Age at Onset Prediction as Early Biomarkers of Brain Atrophy in Huntington's Disease

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Searching brain and peripheral biomarkers is a requisite to cure Huntington's disease (HD). To search for markers indicating the rate of brain neurodegenerative changes in the various disease stages, we quantified changes in brain atrophy in subjects with HD. We analyzed the cross-sectional and longitudinal rate of brain atrophy, quantitatively measured by fully-automated multiparametric magnetic resonance imaging, as fractional gray matter (GM, determining brain cortex volume), white matter (WM, measuring the volume of axonal fibers), and corresponding cerebral spinal fluid (CSF, a measure of global brain atrophy), in 94 gene-positive subjects with presymptomatic to advanced HD, and age-matched healthy controls. Each of the three brain compartments we studied (WM, GM, and CSF) had a diverse role and their time courses differed in the development of HD. GM volume decreased early in life. Its decrease was associated with decreased serum brain-derived-neurotrophic-factor and started even many years before onset symptoms, then decreased slowly in a nonlinear manner during the various symptomatic HD stages. WM volume loss also began in the presymptomatic stage of HD a few years before manifest symptoms appear, rapidly decreasing near to the zone-of-onset. Finally, the CSF volume increase began many years before age at onset. Its volume measured in presymptomatic subjects contributed to improve the CAG-based model of age at onset prediction. The progressive CSF increase depended on CAG mutation size and continued linearly until the last stages of HD, perhaps representing the best marker of progression rate and severity in HD ($R^2 = 0.25$, $P < 0.0001$).

Introduction

Huntington's disease (HD) is caused by an expanded CAG mutation generating a toxic protein named huntingtin that affects cells at several levels within and outside the central nervous system. Mutated huntingtin exerts its main pathological effect on brain neurons. Neuronal dysfunction and degeneration cause progressively invalidating extrapyramidal symptoms, cognitive decline and behavioral changes. Historically, studies on HD neu-

ropathology highlighted as the predominantly affected brain structure the striatum [1], showing progressive striatal dysfunction and degeneration since the beginning of the disease and even before the first symptoms appear [2–4]. Recent reports have nevertheless highlighted widespread cortical and subcortical brain atrophy early in the disease course [4–10].

Precisely, how the mutation influences the rate of brain structure atrophy remains unclear, although progressive neuronal degeneration probably depends on

genetic load [6,9,11]. Additional assumptions on the pathogenesis of HD emphasized the role of glial cells in HD mouse models, cell cultures and patients' brain [12], ascertaining white matter abnormalities and microglial activation since the presymptomatic life stage [4,13–17]. Collectively, the large set of data so far reported on structural changes and neuropathology in HD reflect the heterogeneous clinical features of the illness characterized by the wide variety of symptoms and by the several mechanisms that in concert contribute to the development of disease.

In such a complex scenario, further complicated by the neuropathological and subtle clinical events arising before the first symptoms appear, a crucial need is to identify *in vivo* brain markers (so-called dry biomarkers) of HD staging. The main limitation in searching for brain structural markers was that brain structures had to be traced manually. This technical drawback made it difficult to extend the results worldwide to other centers without incurring errors in reliability. Equally important, automatic or semiautomatic methods for tracing small brain structures such as caudate volumes still need to be validated [18].

Some of these technical problems can be overcome by using a fully automated method for magnetic resonance imaging (MRI) volumetric assessment. Quantitative volumetric measures such as fractional gray matter (fGM, determining brain cortex volume), fractional white matter (fWM, measuring the volume of axonal fibers), and the corresponding fractional cerebral spinal fluid (fCSF, a measure of global brain atrophy), together provide sensitive information about volumes in the various brain compartments calculated by an unsupervised procedure for multiparametric segmentation [19]. Volume loss in the diverse brain tissues as measured by a quantitative MRI procedure therefore provides a sensitive and objective marker of disease progression in various disorders including multiple sclerosis (MS) [20,21] and Alzheimer's disease (AD) [21,22]. This automated procedure also helped to identify and measure the volume of abnormal white matter (aWM) [23,24], due to reactive inflammatory processes, in MS, a neurological disorder mainly involving WM. More research is needed, possibly using an age at onset prediction model [25,26] to characterize better the volumetric changes in brain since the presymptomatic HD stage, so that MRI centers could easily measure them. In future these changes might also prove useful as biomarkers to judge the effectiveness of proposed neuroprotective therapeutic interventions in HD.

In this study, using an unsupervised multiparametric brain segmentation procedure based on an MRI relaxometric approach, we aimed to define the cross-sectional and longitudinal rate of brain atrophy, quan-

titatively measured as fGM and fWM volume loss and relative fCSF volume increase, in a large group of individuals including presymptomatic gene-carriers, patients with advanced HD, and age-matched healthy controls. We also assessed whether the brain atrophy detected on quantitative MRI correlates with the genetic load or the clinical stages of the disease. We then investigated an experimental model to predict age at onset in presymptomatic subjects. To find out whether early degenerative changes are associated with peripheral biological changes we measured brain-derived neurotrophic factor (BDNF) in patients' serum.

Materials and Methods

Subjects

Demographic, clinical and genetic characteristics of subject groups are reported in Table S1. A group of 94 HD mutation carriers (mean age of 41.9 ± 9.8 years, range 14–70; 45 males and 49 females), 35 in the presymptomatic and 59 in the advanced stage of disease, were enrolled in the study and underwent a baseline MRI scan (Table S1). Of these 94 gene carriers, 11 asymptomatic mutation carriers and 21 patients also underwent a delayed MRI examination at least 18 months after the first MRI assessment (mean follow-up interval = 21.32 ± 8.47 months). For control purposes, we used data stored in our data bank from 48 healthy volunteers [4]. All participants underwent genetic testing after informed consent and neurological examination, including motor, psychiatric, cognitive, and functional assessments, by physicians with expertise in HD [6]. Age at onset of symptoms was calculated according to the initial neurological manifestations [6]. Motor symptoms and behavioral and cognitive changes were assessed clinically with the unified Huntington's disease rating scale (UHDRS) [27] and mini-mental state examination (MMSE). Subjects' consent was obtained according to the Declaration of Helsinki (*Br Med J* 1991; 302: 1194). They were assigned to one of seven diagnostic categories to stratify our cohort clinically into subgroups thus distinguishing subjects in a pre-clinical stage of HD from those showing even minimal signs or symptoms of HD: (1) true preclinical HD, no abnormalities, and MMSE above 25; (2) soft signs, abnormal neurological signs despite being specific, insufficient alone to allow a definitive clinical diagnosis of HD, and MMSE above 25; (3) soft signs and symptoms, abnormal neurological signs and symptoms that despite being specific were insufficient alone to allow a definitive clinical diagnosis of HD without the support of molecular genetic testing [27,28] and left the subject's independence and functional capacity unaffected, and MMSE above 25;

(4) manifested HD (stage 1); (5) HD stage 2; (6) HD stage 3; and (7) advanced HD stage 4 or 5. The disease stage was calculated according to the total functional capacity (TFC) score [29]. We considered the subjects with soft clinical manifestations (points 2 and 3) in the so-called zone-of-onset [30]. In subjects with a disease history of at least 5 years, the rate of functional decline and symptom progression was measured in loss of units per year using the TFC and the disability scales (DS) [4,6,29,31]. The DS rating scale combines patients' independence and motor performance, thus taking into account the relationship between independence and neurological motor impairment [31]. Most patients were taking benzodiazepines; some with advanced disease were receiving low doses of atypical neuroleptics (olanzapine, 2.5–10 mg, or risperidone, 1–3 mg), sometimes associated with benzodiazepines, lithium carbonate, or valproate. To highlight decreased biological changes in subjects at the true presymptomatic stage of life and healthy controls, we tested the serum concentrations of BDNF, a growth factor produced by cortical neurons that crosses the brain barrier and that we have already described as lower in the serum of symptomatic HD patients than in controls [32]. Serum BDNF levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BDNF Emax Immunoassay System kit Promega, Madison, WI, USA), according to the manufacturer's instructions. BDNF concentrations in the serum samples were assayed in four independent experiments. Data are presented as means $\pm$ SD.

MRI Acquisition

MRI scans were obtained at a field strength of 1.5 T (GE Signa 1.5 T), sampling the whole brain at 34 levels. The acquisition protocol included 2 interleaved sets of 17 slices covering the entire brain. Each of the 2 sets consisted of 2 conventional spin-echo sequences, generating 15 T1-weighted (TR/TE, 600/15) and proton density/T2-weighted (TR/TE1–TE2, 2,200/15–90) images (25-cm field of view, 256 $\times$ 256 acquisition matrix, 4-mm-thick axial slices). For comparison, we used a neuroimaging archive containing MRI data obtained from healthy controls (Table S1). The MRI control database contained 48 baseline and 15 delayed MRI scans (mean follow-up interval = 15.80 $\pm$ 7.12 months).

MRI Segmentation Procedure and Statistical Analysis

Brain compartments were segmented with a fully automated postprocessing procedure as described else-

where [19]. For each MRI study, total intracranial volume and absolute volumes (in milliliters) of GM, WM, aWM, CSF, the intracerebroventricular volume (ICV), and corresponding fractional volumes (fGM = GM/ICV, fWM = WM/ICV, faWM = aWM/ICV, and fCSF = CSF/ICV), were calculated to allow comparison of data from subjects with different head sizes. For comparison between groups of gene carriers, fGM, fWM, and fCSF were then corrected for age-related changes by adjusting both variables to the mean age of the subjects studied (43.54 years), according to the corresponding rates of yearly decline (0.013%/year for fGM and 0.015%/year for fWM) or of yearly increase (0.15%/year for fCSF), as measured in the MRI database of healthy controls [19]. Two-way analysis of variance (ANOVA) was used to compare groups. Tukey's Kramer multiple comparison test was used to compare differences between each pairs of means with appropriate adjustment for multiple testing. Linear regression was used to assess the relationship between CAG repeat expansion and the rate of whole brain atrophy. Matched-pairs test was used with diagnostic categories with more than one repeated MRI scan, to assess differences in longitudinal measures of brain volume. The over time rate of volume change was calculated for GM, WM, and CSF according to the following formula: Rate of change = ((delayed scan – baseline scan)/baseline scan) $\times$ 100/time between scans. ANOVA with Tukey test was used to compare the mean BDNF values in the groups. *P*-values equal to or less than 0.05 were considered to indicate statistical significance. Statistical analysis was done with JMP version 6 software (SAS Institute Inc., 2005, Cary, NC, USA).

Experimental Model to Predict Age at Onset in Presymptomatic Subjects

To test whether CAG-based prediction of HD age at onset could be improved by assessing fCSF brain volume in presymptomatic subjects, at the time when they underwent MRI, we used a multiple regression approach by taking in account both the expanded CAG repeat number and the fCSF brain compartment volume. From the 35 presymptomatic subjects (Table S1) currently undergoing follow-up, we selected a subset of 23 individuals who, during the longitudinal follow-up, have manifested signs and symptoms of HD. We used the unpaired Student's *t*-test to compare two statistical approaches to calculate the age at onset prediction (taking in account the real age at which the subjects manifested motor symptoms): the simple CAG-based regression model versus the multiple regression model taking in account as variables the CAG repeat number + fCSF volume.

Figure 1 Progressive stage-dependent cerebrospinal fluid compartment increase and dependence on expanded CAG repeat number. A. Axial segmented map of cerebrospinal fluid obtained from baseline and delayed magnetic resonance imaging scan in patients with Huntington's disease (HD). The white-color gain represents the over time volume increase in cerebrospinal fluid and subsequent loss of brain volume, at the various stages of disease. B. Progressive stage-dependent increase in fractional cerebrospinal fluid (fCSF). Volume tissue changes in each disease stage are significant versus controls ($P < 0.0001$), including those seen in preclinical gene carriers. Significant values for the longitudinal fCSF increase are reported in each bar (see also Table 3).

Results

Volumetric Brain-Tissue Changes

Dependence of Volumetric Brain-Tissue Changes on Mutation Size and Clinical HD Stages

UHDRS scores differed significantly between the diagnostic categories of affected subjects (Table S1). The analysis of brain tissues (fGM, fWM, and fCSF), when considering the whole cohort of HD subjects independently from the clinical stage and classification in subgroups (Table S1), showed that the yearly rate of volumetric changes differed in the three brain compartments investigated (for fGM, $F = 2.46$, $P = 0.035$; fWM, $F = 2.08$, $P = 0.07$; and fCSF $F = 4.36$, $P = 0.0013$; ANOVA). The compartment undergoing the greatest volumetric change dur-

ing the HD subjects' life was fCSF, whose relative increase combines the effect of both fGM and fWM loss. The axial segmented map obtained at baseline and delayed MRI scan in HD subjects highlighted a time- and stage-dependent increase in CSF volume (Fig. 1A). The fCSF increase started early, in the presymptomatic stage of life, and progressed through the advanced stages of HD (Fig. 1B). Regression analysis showed that its yearly increase rate measured in followed up subjects depended linearly on the expanded CAG repeat number ($R^2 = 0.24$, $P = 0.0043$, Fig. 2), at longitudinal assessment. Accordingly, the quantitative volumetric assessment of brain tissue changes showed decreased fGM and fWM volumes with a related stage-dependent increase in fCSF (Table 1), at the cross-sectional assessment. The changes in fGM

Figure 2 Linear correlation between yearly increase in fCSF and CAG repeat number in gene-positive preclinical and affected subjects. A positive linear correlation was found between the yearly increase in fCSF and CAG repeat expansion ($R^2 = 0.24$, $P = 0.0043$).

and fWM volumes differed in time course; whereas fGM volume began to diminish already in the “true” preclinical stage of life, well before the first signs and symptoms appeared (Table 1), fWM volume decreased in the “zone-of-onset,” showing progressive volume loss until HD manifested (Table 1). The different time courses were confirmed by both cross-sectional (Tables 1 and 2) and longitudinal (Table 3) analyses. The greatest changes began during the early HD stages: the most prominent change involved the milliliter percentages for fGM loss and the related fCSF increase (Table 2). In agreement with the early decreased fGM volume, serum BDNF mean concentration was already lower in the “true” presymptomatic subjects than in healthy age-matched controls (17019 ± 6977 pg/mL from 22 presymptomatic subjects versus 25318 ± 7019 from 24 control subjects; $P < 0.001$, unpaired *t*-test).

Table 1 Cross-sectional analysis of fractional GM, WM, and CSF volume in healthy control, preclinical, and affected gene-positive subjects, grouped according to their disease stage

Group	n	Mean $\pm$ SD	GM		Mean $\pm$ SD	WM		Mean $\pm$ SD	CSF	
			F	P		F	P		F	P
			41.12	< 0.0001		15.75	< 0.0001		58.92	< 0.0001
Control	48	52.74 $\pm$ 2.9			38.39 $\pm$ 2.20			8.85 $\pm$ 3.13		
True preclinical	22	48.17 $\pm$ 2.31			38.26 $\pm$ 1.99			13.65 $\pm$ 2.71		
Soft signs	10	45.78 $\pm$ 2.39			38.18 $\pm$ 1.87			16.01 $\pm$ 3.29		
Soft signs and symptoms	3	45.67 $\pm$ 0.56			34.48 $\pm$ 1.19			19.79 $\pm$ 0.71		
Stage 1	18	44.46 $\pm$ 3.22			35.28 $\pm$ 2.51			20.13 $\pm$ 3.59		
Stage 2	23	42.13 $\pm$ 3.30			34.62 $\pm$ 2.67			23.11 $\pm$ 4.63		
Stage 3	14	42.44 $\pm$ 3.94			33.39 $\pm$ 3.37			23.96 $\pm$ 5.14		
Stage 4/5	4	42.28 $\pm$ 3.07			31.14 $\pm$ 2.33			26.52 $\pm$ 1.50		

F, ratio of variances for *F*-test between populations.

Table 2 Analysis of differences between groups. The table shows significant differences (*P*) among diagnostic categories on GM, WM, and CSF fractional volumes

Groups			fGM mL	P	fWM mL	P	fCSF mL	P
Control	vs.	true preclinical	4.58	< 0.0001	0.13	ns	4.80	< 0.0001
True preclinical	vs.	soft signs	2.39	< 0.0001	0.08	ns	2.36	0.0903
Soft signs	vs.	soft signs and symptoms	0.11	ns	3.71	0.0209	3.78	ns
Soft signs and symptoms	vs.	stage 1	1.21	ns	0.80	ns	0.34	ns
Stage 1	vs.	stage 2	2.33	< 0.0001	0.66	0.0023	2.98	0.0100
Stage 2	vs.	stage 3	0.31	ns	1.23	ns	0.85	ns
Stage 3	vs.	stage 4	0.16	ns	2.25	ns	2.57	ns

mL, volume difference between groups. All classes show significant differences versus controls. ($P < 0.0001$).

Table 3 Rate and volume change in fGM, and fWM loss and fCSF increase, in healthy controls, and diagnostic categories of gene-positive subjects in the longitudinal study

Diagnostic categories	n	Average follow-up months	GM			WM			CSF		
			Rate of change	(mL)/year loss	P	Rate of change	(mL)/year loss	P	Rate of change	(mL)/year increase	P
Control	15	13	-0.001	-0.01	ns	-0.001	-0.02	ns	0.003	0.05	ns
True preclinical	8	18	-0.001	-0.03	ns	-0.048	-0.87	0.0158	0.014	0.25	ns
Soft signs	3	18	-0.130	-2.34	0.0793	0.001	0.02	ns	0.087	1.56	0.0891
Stage 1	5	19	-0.039	-0.75	0.0437	-0.018	-0.35	ns	0.058	1.11	0.0223
Stage 2	8	20	-0.064	-1.24	0.0465	0.011	0.22	ns	0.051	1.01	0.0141
Stage 3	8	23	-0.050	-1.15	ns	-0.045	-1.04	ns	0.094	2.17	0.0139

The longitudinal analysis of HD subjects and controls showed a variable time- and stage-dependent brain-tissue volume change in HD. Last stage follow-ups are missing due to the small size of the cohort.

Figure 3. Cerebrospinal fluid (fCSF) increase in dependence on disability scale (DS) score per Huntington's disease subjects' stage. A different color marks each stage of the subjects' life including the preclinical stage. Higher fCSF volumes correspond to more severe DS scores and more advanced disease stages according to the total functional capacity scale (regression analysis, $R^2 = 0.25$, $P < 0.0001$).

Correlation of Volumetric Brain-Tissue Changes with Clinical Markers

In the affected subjects ($n = 59$), the single changes in fractional volumes of all the brain compartments analyzed correlated linearly with the decreasing TFC scale score, an index of functional capacity and HD staging (fGM: $R^2 = 0.20$, $P < 0.0001$; fWM: $R^2 = 0.37$, $P < 0.0001$; fCSF: $R^2 = 0.40$, $P < 0.0001$). Similarly, fGM and fCSF correlated linearly with the decreasing DS score, an index including both independence and motor impairment, calculated in subjects subgrouped according to their HD stage (fGM: $R^2 = 0.30$, $P < 0.0001$; fCSF: $R^2 = 0.28$, $P < 0.0001$, Fig. 3). The only MRI variable that correlated linearly with the progressively increasing clinical severity of HD, calculated as a loss of TFC scale score units per year, was the increasing fCSF volume ($R^2 = 0.14$, $P = 0.02$) in patients who had HD for at least 5 years.

Study of Abnormal White Matter

Structural analysis of WM to detect volumes of fractional demyelinated WM due to an inflammatory process [23] highlighted the presence of higher faWM volume in HD subjects with soft signs than in healthy controls (0.081 ± 0.051 vs. 0.017 ± 0.02 , $P < 0.0001$ by ANOVA) and in subjects with true preclinical disease (0.081 ± 0.051 vs. 0.032 ± 0.02 , $P = 0.0003$) (Fig. S1). Increased faWM volumes were higher in subjects with advanced HD than in presymptomatic subjects (0.21 ± 0.20 vs. 0.05 ± 0.04 , $P < 0.0001$) (Fig. S1). Furthermore, faWM volumes were higher in clinical stage 3 than in stage 1 (0.35 ± 0.29 vs.

A

Huntington's disease subjects	n	Median age of symptoms	Predicted median age at onset (CAG-based)	Predicted median age at onset (multiple regression-based)	Age at onset vs standard prediction (p)*	Age at onset vs multiple-regression prediction (p)*
Soft signs	11	41.0	47.0	41.38	0.03	1.00
Manifest symptoms	12	46.0	51.0	47.35	0.05	0.74

*Student t-test

B

Figure 4 (A) Age at onset prediction in HD subjects improved by adding the quantitative fCSF contribute to the CAG-based model. No statistical difference was found between the actual subject's age at onset and the age at onset prediction simulated by our multiple regression approach (CAG + fCSF-based model). (B) Percentage of errors in the prediction of

age at onset. Differences in the percentage of errors in age at onset prediction (y-axis) according to the simple (CAG-based) and multiple regression (CAG + fCSF-based) models (x-axis) in subjects manifesting either soft-sign or clear symptoms of HD.

0.13 ± 0.18 , $P = 0.0068$) and also than in stage 2 (0.35 ± 0.29 vs. 0.21 ± 0.05 , $P = 0.05$).

Predictive Model of Age at Onset

Of 23 presymptomatic subjects who showed clinical manifestations during the longitudinal follow-up, 11 showed soft-signs and 12 signs and symptoms of manifest HD. The median age of the clinical onset manifestations was 41 years for subjects expressing soft signs and 46 for those manifesting clear symptoms of HD (Fig. 4A). The multiple regression approach taking in account both variables, that is, CAG repeat number and fCSF volume, substantially improved the prediction of age at onset based on CAG repeat number alone (41.38 years was the age at onset simulated versus 41 years which represented the real subjects' age at onset). Instead, the model based on expanded CAG repeat number alone simulated the age at onset prediction at 47 years in the same subjects, an age far from the actual onset at 41 years (Fig. 4A). The same multiple regression-approach strategy improved the simulation of onset prediction even in subjects in whom

symptoms of manifest HD developed (Fig. 4A). Moreover, by adding to the CAG repeat number the contribution of fCSF volume to age at onset prediction, the regression coefficient R^2 increased from 0.29 ($P < 0.05$) to 0.74 ($P < 0.01$) in the soft-sign subset of presymptomatic subjects and from 0.83 ($P = 0.0001$) to 0.86 ($P = 0.0001$) in those who manifested clear symptoms of HD, thus substantially diminishing the percentage of errors in age-at-onset prediction (Fig. 4B).

Discussion

The unsupervised multiparametric brain segmentation procedure based on an MRI relaxometric approach we used to examine volumetric changes in HD brain clearly visualized the linear increase in CSF volume during the early to late stages of HD (Fig. 1). Our study therefore confirms and extends previous findings on longitudinal changes in global brain atrophy [8], related to CSF volume increase and to GM and WM volume decrease [4], in patients with HD. Our new findings therefore show that

brain atrophy, as detected by measuring volume changes in fGM, fWM, and fCSF, begins early in the life of gene carriers. Hence, our findings showing that brain WM volume loss, reflecting the altered size and number of dendritic spines [13,33], starts at the presymptomatic stage of life [4,17] (Table 3) and progresses rapidly through the zone-of-onset (Table 2) up to manifest HD suggest that this measure of brain atrophy needs further investigation as a potentially reliable predictor of HD in people who are gene carriers approaching the zone-of-onset, yet near to the probable age at onset of symptoms.

Although the second quantitative measure of brain atrophy we studied, GM volume loss, also starts early in life, before WM volume loss (Table 2), its loss of volume diminishes slowly throughout the disease course and progresses nonlinearly over time towards advanced HD stages (Table 3). Evidence of such early GM volume loss, reflecting the early brain cortex atrophy [4,10], is a novel finding further corroborated by *in vivo* biological changes in the periphery. Indeed, BDNF, a neurotrophic factor produced by cortical neurons [34] implicated in the pathogenic mechanisms of HD [35] and whose levels in serum reflect those in the brain [32,35], already starts to decrease in the presymptomatic stages of HD.

A distinctive, previously unreported finding in our study is that the longitudinal fCSF increase detected in followed-up HD subjects depends strictly on the size of expanded CAG repeat mutation (Fig. 5). This observation is also in line with the large mutation size reported in patients with HD showing particularly severe clinical progression of the disease, probably associated with a faster rate of global brain atrophy than the adult forms carrying moderate mutation sizes [11]. Collectively, our quantitative findings indicate that each of the three brain compartments we studied (WM, GM, and CSF) has a diverse role and their time courses differing in the development of HD (Fig. 4). The GM compartment shows the earliest volume decrease in life, far from manifest HD, thereafter its volume decreases slowly in a nonlinear manner during the various HD stages; WM volume loss begins in the presymptomatic stage of HD, its rapid decrease marking the zone-of-onset. Finally, CSF volume increases many years before age at onset, then continues linearly until the last stages of HD. Measuring its progressive increase could help to improve CAG-based age at onset prediction in unaffected mutation carriers even far from probable manifest HD (Table S1 and Fig. 4). Of these three brain compartments, CSF volume seems also the best predictor of disease progression rate and severity in HD (Figs. 3 and 4), thus confirming that progressive global brain atrophy could be a potential biomarker [8].

Our study has limitations. First, although our large cohort gave us the opportunity to do cross-sectional and

Figure 5 Brain tissue changes in Huntington's disease (HD) subjects throughout life compared with healthy controls. Analysis of 48 healthy control subjects, 35 presymptomatic HD subjects (including "true" presymptomatic, soft sign, and soft sign and symptom subjects) and 59 affected subjects. The cross-sectional volumetric analysis shows an early decrease in fGM volume, a fast fWM loss in the zone-of-onset phase, and a progressive linear increase in the fCSF volume beginning many years before age at onset and continuing until the advanced stages of the disease (fGM: 52.7 ± 2.9 mL in healthy control versus 47.3 ± 2.5 mL in presymptomatic HD versus 42.3 ± 3.5 mL in affected subjects, $F = 134.5$, $P < 0.0001$; fWM: 38.4 ± 2.2 mL in healthy control versus 37.9 ± 2.1 mL in presymptomatic HD versus 34.3 ± 2.9 mL in affected subjects, $F = 41.5$, $P < 0.0001$; fCSF: 8.8 ± 3.1 mL in healthy controls versus 14.8 ± 3.3 mL in presymptomatic HD versus 22.6 ± 4.6 mL in affected subjects, $F = 170.1$, $P < 0.0001$. F, ratio of variances for F-test between populations).

longitudinal analyses, definitive conclusions would need follow-ups of years (in our analysis the longest was 3 years) on a larger sample size, especially the size of the cohort within the zone-of-onset and those at the final HD stages. Enrolling these elderly extremely disabled persons obviously raises practical difficulties. Second, our aim was to search for cerebral markers of HD stages and HD course severity from one stage to another, without taking into account fine regional brain variations. We sought preliminary information on gross brain volume variability in as many HD stages as possible, and used a fully automated procedure that would allow the research to be extended worldwide. Further studies designed to relate fine regional brain changes to the clinical stages and variants of the disease will be a crucial step in disclosing other features in the natural history of HD.

Notwithstanding these limitations, we found that changes in brain volumes differed at the various stages of life. The only marker associated with complete linear

progressive HD brain damage (Fig. 1) and disease progression (Fig. 3) was CSF, whose volumetric increase is a marker of global brain atrophy, as demonstrated in other neurodegenerative diseases [22]. Interestingly, in this study, both WM and GM decreased in a segmental nonlinear manner reflecting the segmental nonlinear trend in the HD progression rate previously described in studies using a clinical approach (Fig. 4) [4,6].

Our observations reflect the major currently accepted pathogenic mechanisms in HD. For example, they support the role of glia and axonal dysfunction and degeneration leading to WM volume loss early in life [4,33]. This early WM volumetric change would substantially contribute to the HD excitotoxic process leaving neurons unprotected against mutant huntingtin, as demonstrated *in vitro* [12]. Again, our findings inferring the occurrence of increasing inflammatory WM tissue in symptomatic people (i.e., demyelinated WM, Fig. S1), reflect the recently published observations on the role of microglial activation correlating with HD severity [15] and beginning early in the life of HD subjects [16]. In their study, the authors performed positron emission tomography procedure [16], by using a tracer specifically detecting activated microglia. Coherently to findings from Tai et al. [16], our data show increased faWM volume beginning since presymptomatic life stage (Fig. S1). Our data also fit in with the evidence that the progression rate in HD depends on the genetic load of the mutation [4,9,11]. Finally, our findings, corroborated by our age at onset prediction model, underline that brain atrophy involving cortical structures begins early, during the presymptomatic stages of HD [4,6], and that early brain degenerative changes are associated with peripheral biological changes (i.e., BDNF in serum), possibly revealing new potential biomarkers in HD. The improved age-at-onset prediction might also help in assessing the benefits of potential neuroprotective therapies, thus favoring novel approaches to be transferred from animal models to humans [36].

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Conflict of Interest

The authors report no conflicts of interest.

References

1. Hersh S, Rosas HD, Ferrante RJ. *Neuropathology and pathophysiology of Huntington's disease*. New York; McGraw-Hill; 2004.
2. Harris GJ, Codori AM, Lewis RF, Schmidt E, Bedi A, Brandt J. Reduced basal ganglia blood flow and volume in pre-symptomatic, gene-tested persons at-risk for Huntington's disease. *Brain* 1999;**122**:1667–1678.
3. Aylward EH, Sparks BF, Field KM, Yallapragada V, Shpritz BD, Rosenblatt A, Brandt J, Gourley LM, Liang K, Zhou H, Margolis RL, Ross CA. Onset and rate of striatal atrophy in preclinical Huntington disease. *Neurology* 2004;**63**:66–72.
4. Ciarmiello A, Cannella M, Lastoria S, Simonelli M, Frati L, Rubinsztein DC, Squitieri F. Brain white-matter volume loss and glucose hypometabolism precede the clinical symptoms of Huntington's disease. *J Nucl Med* 2006;**47**:215–222.
5. Thieben MJ, Duggins AJ, Good CD, Gomes L, Mahant N, Richards F, McCusker E, Frackowiak RS. The distribution of structural neuropathology in pre-clinical Huntington's disease. *Brain* 2002;**125**:1815–1828.
6. Squitieri F, Gellera C, Cannella M, Mariotti C, Cislighi G, Rubinsztein DC, Almqvist EW, Turner D, Bachoud-Lévi AC, Simpson SA, et al. Homozygosity for CAG mutation in Huntington disease is associated with a more severe clinical course. *Brain* 2003;**126**:946–955.
7. Kassubek J, Juengling FD, Kioschies T, Henkel K, Karitzky J, Kramer B, Ecker D, Andrich J, Saft C, Kraus P, et al. Topography of cerebral atrophy in early Huntington's disease: A voxel-based morphometric MRI study. *J Neurol Neurosurg Psychiatry* 2004;**75**:213–220.
8. Henley SM, Frost C, MacManus DG, Warner TT, Fox NC, Tabrizi SJ. Increased rate of whole-brain atrophy over 6 months in early Huntington disease. *Neurology* 2006;**67**:694–696.
9. Ruocco HH, Bonilha L, Li LM, Lopes-Cendes I, Cendes F. Longitudinal analysis of regional gray matter loss in Huntington disease: Effects of the length of the CAG repeat. *J Neurol Neurosurg Psychiatry* 2008;**79**:130–135.
10. Rosas HD, Salat DH, Lee SY, Zaleta AK, Pappu V, Fischl B, Greve D, Hevelone N, Hersch SM. Cerebral cortex and the clinical expression of Huntington's disease: Complexity and heterogeneity. *Brain* 2008;**131**:1057–1068.
11. Rosenblatt A, Liang KY, Zhou H, Abbott MH, Gourley LM, Margolis RL, Brandt J, Ross CA. The association of

- CAG repeat length with clinical progression in Huntington disease. *Neurology* 2006;**66**:1016–1020.
12. Shin JY, Fang ZH, Yu ZX, Wang CE, Li SH, Li XJ. Expression of mutant huntingtin in glial cells contributes to neuronal excitotoxicity. *J Cell Biol* 2005;**171**:1001–1012.
 13. Sapp E, Kegel KB, Aronin N, Hashikawa T, Uchiyama Y, Tohyama K, Bhide PG, Vonsattel JP, DiFiglia M. Early and progressive accumulation of reactive microglia in the Huntington disease brain. *J Neuropathol Exp Neurol* 2001;**60**:161–172.
 14. Rosas HD, Tuch DS, Hevelone ND, Zaleta AK, Vangel M, Hersch SM, Salat DH. Diffusion tensor imaging in presymptomatic and early Huntington's disease: Selective white matter pathology and its relationship to clinical measures. *Mov Disord* 2006;**21**:1317–1325.
 15. Pavese N, Gerhard A, Tai YF, Ho AK, Turkheimer F, Barker RA, et al. Microglial activation correlates with severity in Huntington disease: A clinical and PET study. *Neurology* 2006;**66**:1638–1643.
 16. Tai YF, Pavese N, Gerhard A, Tabrizi SJ, Barker RA, Brooks DJ, Piccini P. Microglial activation in presymptomatic Huntington's disease gene carriers. *Brain* 2007;**130**:1759–1766.
 17. Klöppel S, Draganski B, Golding CV, Chu C, Nagy Z, Cook PA, Hicks SL, Kennard C, Alexander DC, Parker GJ, et al. White matter connections reflect changes in voluntary-guided saccades in pre-symptomatic Huntington's disease. *Brain* 2008;**131**:196–204.
 18. Douaud G, Gaura V, Ribeiro MJ, Lethimonnier F, Maroy R, Verny C, Krystkowiak P, Damier P, Bachoud-Levi AC, Hantraye P, Remy P. Distribution of grey matter atrophy in Huntington's disease patients: A combined ROI-based and voxel-based morphometric study. *Neuroimage* 2006;**32**:1562–1575.
 19. Alfano B, Brunetti A, Covelli EM, Quarantelli M, Panico MR, Ciarmiello A, Salvatore M. Unsupervised, automated segmentation of the normal brain using a multispectral relaxometric magnetic resonance approach. *Magn Reson Med* 1997;**37**:84–93.
 20. Quarantelli M, Ciarmiello A, Morra VB, Orefice G, Larobina M, Lanzillo R, Schiavone V, Salvatore E, Alfano B, Brunetti A. Brain tissue volume changes in relapsing-remitting multiple sclerosis: Correlation with lesion load. *Neuroimage* 2003;**18**:360–366.
 21. Filippi M, Rovaris M, Inglesse M, Barkhof F, De Stefano N, Smith S, Comi G. Interferon beta-1a for brain tissue loss in patients at presentation with syndromes suggestive of multiple sclerosis: A randomised, double-blind, placebo-controlled trial. *Lancet* 2004;**364**:1489–1496.
 22. Fox NC, Freeborough PA, Rossor MN. Visualisation and quantification of rates of atrophy in Alzheimer's disease. *Lancet* 1996;**348**:94–97.
 23. Alfano B, Brunetti A, Larobina M, Quarantelli M, Tedeschi E, Ciarmiello A, Covelli EM, Salvatore M. Automated segmentation and measurement of global white matter lesion volume in patients with multiple sclerosis. *J Magn Reson Imaging* 2000;**12**:799–807.
 24. Tedeschi G, Lavorgna L, Russo P, Prinster A, Dinacci D, Savettieri G, Quattrone A, Livrea P, Messina C, Reggio A, et al. Brain atrophy and lesion load in a large population of patients with multiple sclerosis. *Neurology* 2005;**65**:280–285.
 25. Brinkman RR, Mezei MM, Theilmann J, Almqvist E, Hayden MR. The likelihood of being affected with Huntington disease by a particular age, for a specific CAG size. *Am J Hum Genet* 1997;**60**:1202–1210.
 26. Squitieri F, Sabbadini G, Mandich P, Gellera C, Di Maria E, Bellone E, Castellotti B, Nargi E, de Grazia U, Frontali M, Novelletto A. Family and molecular data for a fine analysis of age at onset in Huntington disease. *Am J Med Genet* 2000;**95**:366–373.
 27. Huntington Study Group. The unified Huntington's disease rating scale: Reliability and consistency. Huntington Study Group. *Mov Disord* 1996;**11**:136–142.
 28. Paulsen JS, Zhao H, Stout JC, Brinkman RR, Guttman M, Ross CA, Como P, Manning C, Hayden MR, Shoulson I, Huntingdon Study Group. Clinical markers of early disease in persons near onset of Huntington's disease. *Neurology* 2001;**57**:658–662.
 29. Marder K, Zhao H, Myers RH, Cudkowicz M, Kayson E, Kiebertz K, Orme C, Paulsen J, Penney JB Jr, Siemers E, Shoulson I. Rate of functional decline in Huntington's disease. Huntington Study Group. *Neurology* 2000;**54**:452–458.
 30. Penney JB Jr., Young AB, Shoulson I, Starosta-Rubenstein S, Snodgrass SR, Sanchez-Ramos J, Ramos-Arroyo M, Gomez F, Penchaszadeh G, Alvir J, et al. Huntington's disease in Venezuela: 7 years of follow-up on symptomatic and asymptomatic individuals. *Mov Disord* 1990;**5**:93–99.
 31. Myers RH, Sax DS, Koroshetz WJ, Mastromauro C, Cupples LA, Kiely DK, Pettengill FK, Bird ED. Factors associated with slow progression in Huntington's disease. *Arch Neurol* 1991;**48**:800–804.
 32. Ciammola A, Sassone J, Cannella M, Calza S, Poletti B, Frati L, Squitieri F, Silani V. Low brain-derived neurotrophic factor (BDNF) levels in serum of Huntington's disease patients. *Am J Med Genet B Neuropsychiatr Genet* 2007;**144**:574–577.
 33. Gutekunst CA, Li SH, Yi H, Mulroy JS, Kuemmerle S, Jones R, Rye D, Ferrante RJ, Hersch SM, Li XJ. Nuclear and neuropil aggregates in Huntington's disease: Relationship to neuropathology. *J Neurosci* 1999;**19**:2522–2534.
 34. Saudou F, Finkbeiner S, Devys D, Greenberg ME. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* 1998;**95**:55–66.
 35. Gauthier LR, Charrin BC, Borrell-Pagès M, Dompierre JP, Rangone H, Cordelières FP, De Mey J, MacDonald

ME, Lessmann V, Humbert S, Saudou F. Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell* 2004;**118**:127–138.

36. Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA, Bland RJ, Young D, Strybing K, Eidelberg D, During MJ. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: An open label, phase I trial. *Lancet* 2007;**369**:2097–2105.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographic, clinical and genetic characteristics of subject groups.

Figure S1. Analysis of fractional abnormal white matter (faWM). Scatter plot of fractional demyelinated WM (faWM in milliliters, x-axis) measured in 48 controls and in 35 asymptomatic subjects and in 59 patients using the unsupervised technique (y-axis), showed a greater faWM volume in patients than in asymptomatic and control subjects ($P < 0.0001$ by unpaired t).

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