The Role of Iron in Gray Matter Degeneration in Huntington's Disease: A Magnetic Resonance Imaging Study

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Abstract: In Huntington's disease, iron accumulation in basal ganglia accompanies neuronal loss. However, if iron content changes with disease progression and how it relates to gray matter atrophy is not clear yet. We explored iron content in basal ganglia and cortex and its relationship with gray matter volume in 77 mutation carriers [19 presymptomatic, 8 with soft symptoms (SS), and 50 early-stage patients) and 73 matched-controls by T2*relaxometry and T1-weighted imaging on a 3T scanner. The ANCOVA model showed that iron accumulates in the caudate in presymptomatic subjects ($P = 0.004$) and remains relatively stable along disease stages in this nucleus; while increases in putamen and globus pallidus ($P < 0.05$). Volume instead decreases in basal ganglia, starting from the caudate ($P < 0.0001$) and extending to the putamen and globus pallidus ($P \leq 0.001$). The longer the disease duration and the higher the CAG repeats, the higher the iron accumulation and the smaller the volume. In the cortex, iron decreases in parieto-occipital areas in SS ($P < 0.027$); extending to premotor and parieto-temporo-occipital areas in patients ($P < 0.003$); while volume declines in frontoparietal and temporal areas in presymptomatic ($P < 0.023$) and SS ($P < 0.045$), and extends throughout the cortex, with the exception of anterior frontal regions, in patients ($P < 0.023$). There is an inverse correlation between volume and iron levels in putamen, globus pallidus and the anterior cingulate; and a direct correlation in cortical structures (SMA-sensorimotor and temporo-occipital). Iron homeostasis is

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

Contract grant sponsor: European Commission, through a Marie Curie Fellowship for Career Development; Contract grant number: FP7-PEOPLE-2011-IEF-301587 (C.S.); Contract grant sponsor: Italian Ministry of Health (IMA); Contract grant number: GRANT 204/GR-2009-1606835 (M.P.); Contract grant sponsor: Italian Ministry of Health (Ricerca Corrente) (F.S.); Contract grant sponsor: Telethon; Contract grant number: GGP12218 (F.S.); Contract grant sponsor: European Huntington's Disease Network for the Registry Study (F.S.)

Disclosure: The authors report no conflicts of interest.

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Received for publication 15 November 2013; Revised 3 July 2014; Accepted 11 August 2014.

DOI: 10.1002/hbm.22612

Published online 21 August 2014 in Wiley Online Library (wileyonlinelibrary.com).

affected in the disease; however, there appear to be differences in the role played by iron in basal ganglia and in cortex. *Hum Brain Mapp* 36:50–66, 2015. © 2014 Wiley Periodicals, Inc.

Key words: Huntington's disease; MRI; iron; neuronal loss; multimodal

INTRODUCTION

Huntington's disease is a neurodegenerative genetic disease determined by a CAG expansion mutation in the gene that encodes the protein huntingtin (Htt). The abnormal function of the mutated Htt causes progressive brain dysfunction and degeneration, which in turn causes a sequence of motor symptoms, neuropsychiatric disturbances and cognitive disorders leading to dementia [Walker, 2007]. The striatum is the most vulnerable brain structure in Huntington's disease, with early pathological alterations consisting of progressive neuronal loss, nuclear inclusions of Htt, inflammation (reactive astrogliosis, microglia, and increased number of oligodendrocytes) and aberrant iron accumulation [Gomez-Tortosa et al., 2001; Vonsattel, 2008].

Iron plays an important role in the brain, it being involved in oxygen transportation, myelin production, synthesis of neurotransmitters (i.e., dopamine and GABA synthesis), and Fenton reaction [Hilditch-Maguire et al., 2000; Langkammer et al., 2010]. Brain iron is mainly stored through binding to ferritin, which serves as a buffer against harmful iron deficiency or iron overload. Ferritin is expressed by neurons, microglia and oligodendrocytes, with oligodendrocytes being those that contain the largest amount [Kell, 2010]. Ferritin-bound iron accumulates in the brain with normal aging until the fourth decade of life, the highest brain iron concentrations typically being found in the basal ganglia [Langkammer et al., 2010]. An increase in iron content has been associated with protein modification, misfolding, and aggregation, leading to the formation of intracellular inclusion bodies; and with pathogenic processes (i.e., inflammation, factor release, morphological change, or apoptosis) [Zecca et al., 2004].

Brain regions involved in motor functions, that is, extrapyramidal regions, tend to have a higher iron content than nonmotor-related regions, which might explain why an iron imbalance is commonly associated with movement disorders [Koeppen, 2003; Zecca et al., 2004]. Indeed, increased iron deposition in the basal ganglia has been associated with tissue damage in several neurological disorders, including Parkinson's disease, Alzheimer's disease and multiple sclerosis [Koeppen, 2003; Langkammer et al., 2010; Péran et al., 2010; Zecca et al., 2004]. Iron metabolism is also known to be altered in Huntington's disease. Gene knockdown models have shown that oxidation, localization and structural organization of the inclusion bodies formed by mutant *htt* are iron-dependent [Kell, 2010; Lumsden et al., 2007].

Iron content in the brain can be measured by means of MRI. The variations in the magnetic susceptibility of brain tissue may have different biophysical origins: in the gray matter, magnetic susceptibility is originated above all from ferritin-bound iron, whereas in the white matter it is also affected by myelin. Recent advances in MRI technology and image analysis methods have led to the development of a technique that can be used to map brain areas characterized by their iron content: T2*-weighted imaging (relaxation time mapping or relaxometry) [Langkammer et al., 2012, 2010; Péran et al., 2009, 2007]. Relaxometry provides a reliable means of studying iron distribution in the brain since relaxation rate ($R2^*$) maps show a strong linear correlation with chemically determined iron concentrations, particularly in gray matter structures [Langkammer et al., 2012].

The few MRI studies conducted to assess iron levels on Huntington's disease have confirmed the presence of increased iron content in the caudate, putamen and globus pallidus from as early as the presymptomatic stage by means of different techniques [Bartzokis et al., 1999, 2007; Dumas et al., 2012; Rosas et al., 2012; Sánchez-Castaneda et al., 2013; Vymazal et al., 2007]. However, differences in iron accumulation in the basal ganglia between presymptomatic and Huntington's disease patients have not yet been explored.

Volumetric studies have consistently revealed progressive striatal (caudate and putamen) atrophy long before the onset of motor symptoms [Aylward, 2007; Aylward et al., 2011a, 2011b, 2012; Tabrizi et al., 2009, 2011, 2012; Paulsen et al., 2006, 2010; Rosas et al., 2003, 2005]. Nevertheless, whether iron accumulation is independent of or is related to other neuropathological processes, such as gray matter loss, is not yet clear [Dumas et al., 2012; Sanchez-Castaneda et al., 2013]. Understanding the timing of iron mismanagement in relation to the progression of neuronal loss may provide a deeper insight into the pathogenesis of Huntington's disease, and raise the possibility of monitoring iron changes as a complementary marker of disease progression.

Although striatal atrophy is a widely used marker of Huntington's disease [Aylward, 2007], the cortical involvement in the early stages of the disease still remains unclear. Recent studies have described reduced volume and thinning of cortical areas, prevalently in posterior regions (i.e., precentral and superior frontal, temporal, parietal and occipital areas) [Aylward et al., 2011a, 2011b; Paulsen et al., 2006, 2010; Rosas et al., 2005, 2012; Tabrizi et al., 2009]. Also, very recently Rosas et al. [2012]

described cortical iron disruption in advanced Huntington's disease patients (stage III) in similar regions, confirmed by postmortem spectrometry. They showed an increase in iron content in the frontal and parietal cortex and anterior cingulate, and a decrease in iron content in occipital areas. However no studies have yet evaluated the relationship between cortical atrophy and iron changes in Huntington's disease since presymptomatic stages.

All the aforementioned findings indicate that iron causes local changes in magnetic susceptibility, an effect that can be measured by means of MRI using relaxometry. We therefore used: (i) R2* relaxometry to quantify iron deposition in the gray matter of a large cohort of Huntington's disease subjects, focusing on any differences between clinical groups both at the level of basal ganglia and whole-brain cortex; (ii) T1 3D volumetry to quantify gray matter volume changes in basal ganglia and whole-brain cortex; and (iii) to investigate the relationship between iron concentrations and gray matter atrophy. We also studied the relationship between the number of CAG repeats, the iron accumulation and volume decrease.

MATERIALS AND METHODS

Subjects

Ninety-four mutation carriers (CAG repeats ≥ 39) were enrolled in the study. Seven subjects were unable to finish the MRI scanning protocol, while 10 were excluded because of artifacts in the MRI images that prevented pre-processing. All of the excluded subjects were symptomatic. There were not statistically significant differences between the drop-out group and the included HD patients, even if they might have slightly lower cognitive punctuations and more motor and functional impairment. Thus, the final patient sample was composed of 77 mutation carriers and 73 age- and gender-matched control subjects. All the subjects were clinically examined by the same neurologist (F.S.), who is an expert in Huntington's disease, using the unified Huntington's disease rating scale (UHDRS) motor, cognitive, behavioral and functional subscales [Huntington Study Group, 1996], and the mini-mental state examination (MMSE) for the general cognitive assessment [Folstein et al., 1975]. The cohort was then subdivided into 19 presymptomatic subjects (PreHD), 8 individuals with soft symptoms (SS) and 50 symptomatic subjects (HD) in the early stages (I-II). PreHD were defined on the basis of a total UHDRS motor score of <5 and a cognitive and behavioral assessment within the normality; while SS were defined on the basis of suspicious clinical features that were insufficient for a diagnosis of Huntington's disease [Paulsen et al., 2001]. In all SS cases showing a motor score above 5, the chorea score (that is the most visible symptom) was very low and not exceeding 7, that would be the result of having a score 1 in each of the symptoms of the subscale. The cognitive evaluation included the phonetic

verbal fluency test, the symbol digit test, the Stroop test and the MMSE. The MMSE score, corrected for age and education, was within the normal range for all the PreHD and SS subjects. The rest of the cognitive measures were within the mean and 1.5 standard deviation in comparison with previously published normative data [Paulsen et al., 2001]; except the symbol digit in both PreHD and SS and the Stroop-reading in SS. Symbol digit requires elements of attention, visuo-perceptual processing, working memory, and psychomotor speed and it is sensitive to impairments in premanifest HD, especially in individuals who are closer to expected diagnosis [Stout et al., 2011]; probably due to its sensitivity to the psychomotor slowness that characterizes the disease. Furthermore, slowness in Stroop-reading might be related to brady-psychia or lower cultural level than the normative sample. None of the PreHD or SS individuals showed significant behavioral changes and, according to Brinkman et al. [1997], they were not showing significant psychiatric disturbances altering the normal state of the life. The patients' age at disease onset was retrospectively established by both interviews to family members and patients and by the analysis of clinical files (i.e., motor, cognitive, and behavioral assessment), aimed at determining the first motor symptoms and cognitive/psychiatric abnormalities that represented a permanent change from the normal state [Brinkman et al., 1997, Squitieri et al., 2003]. The predicted years to manifest disease were calculated on the basis of the survival analysis formula described by Langbehn et al. [2004]. Furthermore, in an attempt to estimate the progression of the pathological process from the presymptomatic stage, we calculated a 'HD development' index by combining the predicted years to onset for PreHD subjects and disease duration (years from onset) for patients [Di Paola et al., 2012; Sanchez-Castaneda et al., 2013]. The disease burden index was measured according to the previously described formula: $\text{age} \times (\text{CAG} - 35.5)$ [Penney et al., 1997]. Patients were excluded if they were in the advanced stages of disease (stages III-IV) or had MRI focal lesions. The demographic and clinical characteristics of the sample are shown in Table I. Our local ethics committee approved the study and written informed consent was obtained from all the participants.

MRI Acquisition

All the participants were examined using a 3 Tesla Allegra scanner with a standard quadrature (Siemens Medical Solutions). The MRI protocol included whole-brain T2*-weighted and T1-weighted sequences. All sequences were obtained on the sagittal plane along the AC/PC line. Six consecutive T2*-weighted gradient-echo volumes were acquired using a segmented echo-planar imaging sequence at different TEs: 6, 12, 20, 30, 45, and 60 ms (TR = 5000; bandwidth = 1116 Hz/vx; matrix size 128×128 ; 80 axial slices; flip angle 90° ; voxel size 1.8 mm^3); whole-brain T1-weighted images were obtained by means of a modified

TABLE I. Demographic and clinical characteristics of the sample (clinical subgroups)

	Controls (<i>n</i> = 73)	PreHD (<i>n</i> = 19)	SS (<i>n</i> = 8)	HD (<i>n</i> = 50)	χ^2/t	<i>P</i> -value
Gender (M:F)	43:30	11:8	8:0	30:20	5.35	NS
Age	44.5 (13.0)	36.3 (6.5)	42.7 (7.3)	48.1 (13.4)	4.3	0.013 ^a ; 0.001 ^{c++}
CAG Repeat Length	NA	42.7 (0.6)	44.2 (2.5)	45.7 (5.4)	4.3	0.006 ^{c++}
Disease duration (years)	NA	NA	NA	7.81 (4.7)	NA	NA
TIV	1413450 (136018.75)	1389361 (123010.86)	1436319 (130281.3)	1342613 (127281.2)	3.2	0.023 ^{b++}
MMSE	NA	28.5 (1.4)	27 (1.1)	24.4 (3.9)	10.7	<0.0001 ^d ; 0.04 ^{g++}
UHDRS Motor	NA	2.7 (1.8)	12 (4.8)	38.4 (14.9)	50.5	<0.0001 ^{cftt}
UHDRS Cognitive	NA	270.5 (41.2)	240 (42.5)	134.6 (46.3)	55.2	<0.0001 ^{dgtt}
UHDRS Behavioral	NA	8.93 (8.8)	4.13 (4.9)	18.36 (9.1)	13.4	<0.0001 ^{cftt}
UHDRS Functional	NA	25 (0)	25 (0)	17.5 (6)	22.7	<0.0001 ^{dgtt}
TFC	NA	13 (0)	13 (0)	8.1 (2.6)	59.2	<0.0001 ^{dgtt}
Independence scale	NA	100 (0)	100 (0)	77.2 (13.4)	42.3	<0.0001 ^{dgtt}
Disease burden	NA	261.1 (60.9)	349.8 (34)	454.8 (117.3)	11.9	<0.0001 ^c ; 0.04 ^{ftt}

Values expressed as mean (S.D.) with the exception of gender.

[†]Pearson's Chi-square

⁺⁺T-student. Bonferroni correction.

HD, Huntington's disease; MMSE, mini-mental state examination; NA, not applicable; NS, not significant; PreHD, presymptomatic Huntington's disease; SS, soft symptoms; TIV, total intracranial volume; TFC, total functional capacity

^aPreHD < Controls.

^bHD < Controls.

^cPreHD < HD, lower punctuations mean better performance.

^dPreHD > HD.

^ePreHD < SS, lower punctuations mean better performance.

^fSS < HD, lower punctuations mean better performance.

^gSS > HD.

driven equilibrium Fourier transform (MDEFT) sequence (TE/TR = 2.4/7.92 ms, flip angle 15°, voxel size 1 mm³).

MRI Data Processing

Image processing was performed by combining FSL 4.1(www.fmrib.ox.ac.uk/fsl/), SPM8 (<http://www.fil.ion.ucl.ac.uk/spm/software/spm8/>) running in Matlab (vers.7.9, the MathWorks) and Freesurfer (<https://surfer.nmr.mgh.harvard.edu/>).

Whole-brain analysis of iron content (Voxel-based relaxometry)

The first echo of the T2* weighted volume series (TE 6) had the highest contrast to noise ratio (CNR). The five other echoes were therefore registered to the first echo with a 12-parameter affine transformation and then averaged. A full-affine 3D alignment was then calculated between each of the six T2*-weighted volumes and the mean T2*-weighted volume. As a result of this processing, T2*-weighted volumes were corrected for head movements. For each subject, we performed a voxel-by-voxel nonlinear least-squares fitting of the data acquired at the six TEs to obtain a monoexponential signal decay curve ($S = S_0 e^{-t/T_2^*}$). In order to facilitate the analysis of the relaxation results, we considered the inverse relaxation times (i.e., relaxation rates

$R_2^* = 1/T_2^*$). Artifacts in the susceptibility maps present in very superficial areas of the cortex close to air sinuses (notably in the orbitofrontal cortex) were excluded by applying a brain mask defined on the first echo (TE6), which had the highest CNR (see Supporting Information Fig. 2). The resulting skull-stripped volume was virtually artifact-free and registered with an affine transformation to the skull-stripped anatomical T1 scan with FSL FLIRT [Jenkinson et al., 2002]. Then, a nonlinear registration between whole skull volumes, initialized with the previous linear registration, was performed with FSL FNIRT to provide the "T2* to T1" registration. The same procedure was applied to register the subject T1 to the MNI T1 template and resulted in the calculation of the "T1 to MNI T1" transformation. Finally, combining the "T2* to T1" and "T1 to MNI" registrations allowed to estimate the "T2* to MNI" transform which was applied to all T2* maps. Prior to postprocessing all images were screened for artifacts and clinical abnormalities by a clinical neuroradiologist (U.S). Finally, to perform the statistical analysis, all the individual T2* maps were smoothed using a full width at half maximum (FWHM) Gaussian kernel of 6 mm. Differences in regional iron concentration between groups were assessed using the full factorial design implemented in SPM8 with one fixed factor (clinical group) including age, gender and total intracranial volume (TIV) as covariates to control for their effect. We performed first whole-brain comparisons; and second, we selected three subcortical regions of interest (ROIs), the

caudate, putamen, and globus pallidus bilaterally. The ROIs were automatically traced using the Pick Atlas tool version 3.0.3 from the SPM package to restrict the statistical analysis to the regions contained in the mask file. Thus, we performed a small volume correction that was reflected in the P -values. For all the statistical analyses, the threshold was settled at voxel and cluster levels $P < 0.05$ family-wise error (FWE) corrected for multiple comparisons.

Whole-brain analysis of gray matter volume (Voxel-based morphometry)

Images were processed and analyzed using Voxel-based morphometry (VBM) [Ashburner and Friston, 2000; Good et al., 2001] in the statistical parametric mapping framework (SPM8, Wellcome Department of Imaging Neuroscience, University College London, UK). VBM detects differences in the regional concentration of gray matter at a local scale after discounting global differences in anatomy and position. Each MDEFT volume was segmented first into gray matter, white matter and cerebrospinal fluid. Then, the DARTEL toolbox was then applied to the partitions [Good et al., 2001]. The pre-processing steps included creating a template, normalization of the images to that template, modulation with Jacobian determinants, and smoothing of the gray matter maps with an isotropic 6 mm FWHM Gaussian kernel following previous published methods [Ashburner and Friston, 2000; Good et al., 2001]. Differences in gray matter between groups were assessed by the full factorial design implemented in SPM8 with one fixed factor (clinical group) including age, gender and TIV as covariates to control for their effect. We performed a whole-brain comparison. For all the statistical analyses, the threshold was settled at voxel and cluster levels $P < 0.05$ FWE corrected for multiple comparisons. The result is a statistical parametric map that highlights regions in which gray matter concentrations differ between groups.

Freesurfer analysis

To perform the correlation analysis, we further calculated the individual gray matter volume and mean $R2^*$ of the caudate, putamen and globus pallidus and cortical structures using Freesurfer (<http://ftp.nmr.mgh.harvard.edu/>). The version of Freesurfer used was the X86_64 Red-Hat running in a linux gnu stable v5.2. We used the default parameters to perform subcortical segmentations (aseg) and cortical parcellations (aparc). We also calculated the TIV to be used in all the previous voxel-based analysis as confounding factor. Based on these segmentations, the absolute volume of each structure (caudate, putamen, globus pallidus and cortical gyri) was calculated with Freesurfer. Then, $T2^*$ images were registered to the $T1$ -weighted images in a two-step way: first linearly registered to the $T1$, and then, nonlinearly to the $T1$ in MNI. The segmentation masks could then be registered to the $T2^*$ maps. Averaged intensity values were calculated with

FSLstats for each segmented structure bilaterally. For basal ganglia, we calculated an average right/left volume and mean iron content for each nucleus. Finally, the same way as with the VBM/VBR, we perform a full factorial design with one fixed factor (clinical group) and age and gender as covariates for the Freesurfer extracted-volumes and mean iron content with SPSS (15.0, SPSS).

Statistical analysis

Differences between clinical groups were assessed by two ANCOVA models: first, to test the differences in iron content and gray matter volume between disease staging groups; we included the clinical group as factor (PreHD, SS, and HD), and age, gender and TIV as covariates. Second, to test the influence of the CAG repeats length on iron accumulation and gray matter volume, we divided mutation carriers into three groups: (1) low-CAG repeats, with repeats between 39 and 44; (2) medium-CAG repeats, with repeats between 45 and 49; and (3) high-CAG repeats, with repeats over 50. We then performed an ANCOVA model with the CAG group as factor and age, gender and TIV as covariates. The results of both ANCOVA models were corrected for multiple comparisons using a FWE rate correction set at $P < 0.05$, when performed with SPM8; and with a post-hoc t -student contrast with Bonferroni's correction, when made by SPSS (15.0, SPSS). The X^2 test was used for qualitative variables.

For the correlation analyses, we used Pearson's correlation test (15.0, SPSS). The effect of age, gender and TIV was controlled by including them as covariates in all the comparisons.

RESULTS

Demographic and Clinical Characteristics of the Sample

PreHD and HD patients differed in age and CAG repeat length; PreHD were also younger than controls (Table I). Moreover, as expected, HD patients yielded significantly poorer performances in all the clinical measures and significantly higher disease burden scores than PreHD and SS subjects. There were no significant clinical differences between PreHD and SS. Regarding the CAG groups, the higher the CAG repeats, the higher the impairment in motor, cognitive, and functional scales (Table IV).

Basal Ganglia

Differences in mean relaxation rates ($R2^*$)

Voxel-based relaxometry. When compared with controls, higher iron levels were found in PreHD subjects in the left caudate ($P = 0.004$), in SS subjects in the left putamen and globus pallidus bilaterally ($P = 0.006$ left, $P = 0.033$ right) and in HD patients in the putamen and globus pallidus

Figure 1.

Basal ganglia. **(A)** Volume and mean R2* values of the caudate, putamen and globus pallidus in the three clinical groups and controls (with the 95% confidence interval). The blue dots represent the volume and the red dots the R2* mean value. The lines represent the 95% confidence interval. **(B)** Stereotactic locations of

the areas of R2* increase in clinical groups compared to controls (age, gender and TIV as covariates; whole-brain analysis, $P < 0.05$ FWE). **(C)** Stereotactic locations of areas of significant volume loss in clinical groups compared to controls (age, gender and TIV as covariates; whole-brain analysis, $P < 0.05$ FWE).

bilaterally ($P < 0.0001$) (Table II, Fig. 1b). When the clinical groups were compared, iron levels in PreHD subjects were higher than in SS subjects in the left caudate ($P = 0.011$), and in the caudate bilaterally than HD patients ($P < 0.0001$); but lower than HD patients in the putamen/globus pallidus bilaterally ($P < 0.037$) (Table II). These results improved when we performed a ROI volume correction: PreHD subjects had significant higher iron content in the left caudate, putamen and globus pallidus than controls; iron content was even higher in SS subjects bilaterally, and to an even greater extent in HD patients, in the putamen and globus pallidus bilaterally.

Freesurfer analysis. Freesurfer segmentations showed that, when compared with controls, all patient groups had increased iron levels in the putamen and globus pallidus ($P < 0.05$) (Table III, Fig. 1a); while in the caudate nucleus there was a trend toward significance, especially in PreHD subjects ($P = 0.08$). When the clinical groups were compared, PreHD subjects had higher iron levels in the cau-

date than HD patients ($P = 0.028$) and lower iron levels in the globus pallidus than SS and HD patients ($P = 0.06$ and 0.044 respectively). When the iron accumulation per volume ratio was calculated, iron levels in all three structures in all the clinical groups were found to be higher than in controls, with levels significantly increasing as the disease progressed (Table III, Supporting Information Fig. 1).

Iron concentration differences between CAG groups. When compared with controls, all the CAG groups had higher iron levels in the putamen ($P \leq 0.009$) and globus pallidus ($P < 0.001$) (Fig. 2, Table IV). When the clinical groups were compared, patients with a higher number of CAG repeats (≥ 45) had more iron in the globus pallidus bilaterally than patients with a lower and medium CAG repeats (≤ 44) (SPM: *right*: $P = 0.031$ $t = 5.39$; *left*: $P = 0.040$ $t = 5.25$; Freesurfer: $P = 0.039$ and 0.031). When the iron accumulation per volume ratio was calculated (R2*/volume ratio), iron levels in all three structures in all the clinical groups were found to be higher than in controls.

TABLE II. Stereotactic locations and Brodmann areas (BA) of significant differences between clinical groups and controls (VBR analysis including age, gender and TIV as covariates)

Region (BA)	Cluster (<i>P</i> corrected)	Cluster size (mm ³)	MNI coordinates (<i>x, y, z</i>)	<i>t</i> -value ^a
Control < PreHD				
L Caudate	0.004	25	-18 0 24	5.20
Control < SS				
L Putamen/Globus Pallidus	0.006	34	-22 0 2	5.60
R Globus Pallidus	0.033	3	14 0 0	4.87
Control < HD				
R Putamen	<0.0001	424	22 6 2	8.73
R Globus Pallidus			12 -2 -2	8.27
L Globus Pallidus	<0.0001	440	-12 2 -2	8.58
L Putamen			-22 2 2	8.55
Control > PreHD				
	NS	NS	NS	NS
Control > SS				
L Cuneus (17)	0.006	19	-24 -78 28	5.57
L inferior Parietal (40)	0.027	5	-40 -52 50	5.15
Control > HD				
R Premotor cortex (6)	0.011	12	20 -12 66	5.25
L middle Frontal	<0.0001	151	-38 22 4	6.77
L middle Frontal	<0.0001	69	-28 58 6	5.75
L superior Frontal	0.003	31	-22 40 30	5.58
R Insula (13)	<0.0001	41584	40 -20 8	12.13
L Insula (13)	<0.0001	901	-42 8 26	8.65
R Parahippocampal gyrus	0.001	44	24 -62 -10	5.51
L middle Temporal (37)	0.003	32	-52 -22 -6	5.46
L superior Temporal	0.011	12	-54 -40 4	4.98
R Supramarginal gyrus (40)	0.021	12	58 -48 34	5.07
L inferior Parietal (40)	0.010	13	-64 -36 26	5.51
L Lingual gyrus (19)	<0.0001	174	-20 -84 -10	6.56
R middle Occipital (18)	<0.0001	256	46 -68 2	6.30
PreHD > SS				
L Caudate	0.017	7	-18 4 24	4.90
PreHD > HD				
L Caudate	<0.0001	8954	-14 -4 20	9.02
L Putamen			-22 9 6	18.95
R Caudate			14 -16 18	8.94
L anterior Cingulate (33)	0.017	7	-4 20 24	5.03
L Precentral gyrus	<0.0001	1067	-46 -16 36	7.55
R Precentral gyrus	<0.0001	377	42 -14 38	7.1
R Premotor (6)	<0.0001	519	24 2 50	6.67
L Premotor (6)	0.016	13	-46 2 38	5.38
R Paracentral (6)	0.009	14	8 -32 70	5.46
R inferior Frontal	<0.0001	470	46 10 30	6.78
L superior Frontal	0.003	31	-28 58 4	5.54
L middle Frontal	<0.0001	421	-26 -10 50	7.29
L Insula (13)	<0.0001	757	-44 -16 4	7.53
L middle Temporal gyrus	0.004	25	-54 -56 16	5.40
R middle Temporal gyrus	0.002	34	46 -66 20	5.39
L Hippocampus	0.003	30	-20 34 0	5.55
L Postcentral (3)	<0.0001	105	-26 -42 52	5.94
R Postcentral (3)	0.002	50	24 -40 58	5.55
R inferior Parietal	<0.0001	144	34 -52 42	6.31
R Supramarginal (40)	0.008	29	58 -48 34	5.44
L middle Occipital (18)	0.004	24	-42 -70 4	5.67
R middle Occipital (18)	<0.0001	10346	30 -70 28	8.53

TABLE II. (continued).

Region (BA)	Cluster (<i>P</i> corrected)	Cluster size (mm ³)	MNI coordinates (x, y, z)	<i>t</i> -value ^a
PreHD < HD				
R Putamen	0.003	29	22 6 2	5.58
R Globus Pallidus	0.037	5	10 -2 -2	4.89
L Putamen	0.007	18	-20 6 0	5.36
L Globus Pallidus	0.007	17	-10 -2 -2	5.50
SS > HD				
R Thalamus	0.013	10	16 -16 16	5.06
Anterior Cingulate	0.007	18	4 22 24	5.63
L posterior Cingulate (30)	<0.0001	108	-6 -52 28	5.65
R Premotor (6)	<0.0001	72	2 -8 52	5.97
L Premotor (6)	<0.0001	121	-50 -10 26	5.81
L SMA	0.008	16	-2 -18 54	5.49
L middle Frontal	0.009	14	-26 -10 50	5.37
L Precentral	0.017	7	-46 0 38	5.10
R inferior Frontal	0.016	8	44 12 30	4.93
R superior Temporal (22)	0.0102	41	50 -14 4	5.76
R middle Temporal	0.006	20	44 -68 20	5.49
R Precuneus	0.007	17	18 -62 22	5.14
R middle Occipital	0.002	37	30 -70 28	5.70
R superior Occipital	0.011	12	22 -88 22	5.28

^aSignificance threshold $P < 0.05$ voxel-level corrected for multiple comparisons (FWE)

Differences in gray matter volume

Voxel-based morphometry. When compared with controls, PreHD subjects displayed a reduction in the caudate volume bilaterally ($P < 0.001$) and in the left putamen ($P = 0.018$); SS subjects had reduced caudate and putamen volumes bilaterally ($P < 0.001$); and HD patients had reduced caudate, putamen and globus pallidus volumes bilaterally ($P < 0.001$) (Table V, Fig. 1c). When the clinical groups were compared, SS subjects and HD patients had smaller caudate, putamen and globus pallidus volumes bilaterally than PreHD; extending in the HD patients the impairment to the right thalamus. With ROIs volume correction, nuclei present a pattern of significant volume loss: HD < SS < PreHD.

Freesurfer analysis. Freesurfer analysis (Table III, Fig. 1a) showed reduced volume in all three nuclei bilaterally from the PreHD stages ($P < 0.001$). All nuclei present a pattern of significant volume loss: HD < SS < PreHD.

Gray matter volume differences between CAG groups. The Freesurfer analysis (Table IV, Fig. 2a) revealed a volume reduction in all three nuclei in all the CAG groups compared to controls ($P < 0.001$); the reduction in volume was proportional to the increase in the number of CAG repeats, with a pattern of lower volume, the higher the CAG length: over 45 repeats < 43–44 repeats < below 42 repeats.

Cortical Structures

Iron content

Voxel-based relaxometry. When compared with controls, PreHD subjects did not display any changes in iron con-

tent in cortical areas; SS subjects had significantly reduced R2* signal in the left cuneus (BA 17) and inferior parietal areas (BA 40); and HD patients in the right premotor cortex (BA 6), left frontal areas, insula bilaterally (BA 13), right parahippocampal gyrus; left temporal areas (BA 37), and in the parietal (BA 39,40) and occipital cortices bilaterally (BA 18,19) (Table II, Fig. 3a).

When the clinical groups were compared, HD patients had lower iron levels than PreHD subjects in the anterior cingulate (BA 33), in premotor and SMA cortex bilaterally (BA 6), in sensorimotor (BA 3) and parietal areas bilaterally (BA 7,40), in the left insula (BA 13) and hippocampus, in the bilateral temporal (BA 41), and in the occipital regions bilaterally (BA 18–19). HD patients had also lower iron levels than SS subjects in the anterior and posterior cingulate (BA 30–33), in the premotor and SMA areas bilaterally (BA 6), and in the right temporal (BA 22) and occipital areas (Table II).

Freesurfer analysis. Freesurfer cortical parcellations showed that compared to controls, PreHD subjects did not display any changes in iron content in cortical areas; SS subjects had significant reduction in iron content in the left precuneus and the pericalcarine region; and HD patients have reduced iron content in the posterior cingulate, the middle and superior frontal bilaterally, sensorimotor regions bilaterally (precentral, paracentral, and postcentral gyri); the left supramarginal gyrus, the inferior and superior parietal bilaterally; precuneus and cuneus bilaterally; the left fusiform gyrus, parahippocampus and superior temporal bilaterally; and the pericalcarine, lingual and lateral occipital gyri bilaterally ($P < 0.05$ Bonferroni corrected) (Fig. 3b; Supporting Information Fig. 3).

Figure 2.

CAG groups. **(A)** Volume and mean R2* values of the caudate, putamen and globus pallidus in the three CAG groups and controls (with the 95% confidence interval). The blue dots represents the volume and the red dots the R2* mean value. The

lines represent the 95% confidence interval. **(B)** Stereotactic locations of the areas of R2* increase in all the CAG groups compared to controls (age, gender, and TIV as covariates; whole-brain analysis, $P < 0.05$ FWE).

Cortical volume

Voxel-based morphometry. When compared with controls, a reduction in gray matter volume was observed: bilaterally in frontal and premotor regions (BA 6,8,9,11), parietal and sensorimotor areas (BA 3,7,40), and temporal areas in PreHD subjects; bilaterally in frontal and premotor regions (BA 6,9,47), left sensorimotor areas (BA 3) and inferior temporal in SS subjects; and in the posterior cingulate, frontal and premotor regions bilaterally (BA 6,9,47), right insula (BA 13), right parahippocampus, and bilaterally in medial temporal, parietal (7,40) and occipital areas (BA 19,17) in HD patients (Table V, Fig. 3a).

Freesurfer cortical volume analysis. Freesurfer cortical parcellations showed that compared to controls, PreHD have significant volume reduction in the left orbitofrontal and superior frontal gyri, the left entorhinal gyrus and the right inferior parietal; SS subjects had a significant reduction in the precentral and superior parietal gyri bilaterally,

left entorhinal gyrus, left cuneus and right lateral occipital, and pericalcarine area bilaterally. While HD patients have decreased volume in almost all cortical regions: posterior cingulate and fronto-temporo-parietal and occipital areas bilaterally ($P < 0.05$ Bonferroni corrected) (Fig. 3b). Furthermore, HD patients have volume reductions in the posterior cingulate, frontal, temporal, parietal, and occipital regions compared to PreHD subjects; and in the left orbitofrontal, postcentral gyrus, inferior parietal, and precuneus; bilateral paracentral, supramarginal and lingual gyri; and right middle and superior temporal gyri compared to SS ($P < 0.05$ Bonferroni corrected).

Correlations between iron concentrations and volume

In basal ganglia, the correlation analyses revealed a significant inverse correlation between iron concentrations

TABLE III. ANCOVA with clinical groups including age and gender as covariates

	Controls (<i>n</i> = 73)	PreHD (<i>n</i> = 19)	SS (<i>n</i> = 8)	HD (<i>n</i> = 50)	<i>X</i> ² / <i>T</i>	Compared to controls (<i>P</i> -value)	Differences between groups (<i>P</i> -value)
Caudate R2*	18.93 (2.34)	19.56 (1.65)	19.42 (1.46)	18.77 (3.67)	1.73	NS ^{abc}	0.028 ^e
Putamen R2*	24.91 (2.72)	25.28 (2.16)	27.54 (2.03)	27.7 (4.03)	8.1	0.05 ^a , 0.017 ^b , <0.0001 ^c	NS ^{def}
G. Pallidus R2*	34.14 (4.12)	36.52 (4.18)	40.91 (5.04)	40.72 (6.51)	17.26	0.01 ^a , 0.001 ^b , <0.0001 ^c	0.06 ^d , 0.044 ^e
Caudate volume	3478.38 (401.76)	2946.76 (522.89)	2417.56 (358.54)	1989.43 (495.14)	112.49	<0.0001 ^{abc}	0.002 ^d , <0.0001 ^e , 0.036 ^f
Putamen volume	5637.23 (830.44)	5131.52 (875.56)	4012.75 (710.46)	2977.01 (624.04)	136.19	0.001 ^a , <0.0001 ^{bc}	<0.0001 ^{de} , 0.008 ^f
G. Pallidus volume	1638.36 (223.15)	1425.21 (287.77)	1171.5 (131.18)	963.96 (163.82)	108.7	<0.0001 ^{abc}	0.001 ^d , <0.0001 ^e , 0.06 ^f
Ratio R2*/vol Caudate	0.55 (0.08)	0.68 (0.15)	0.82 (0.15)	1 (0.31)	47.73	0.007 ^a , <0.0001 ^{bc}	<0.0001 ^e ; 0.05 ^f
Ratio R2*/vol Putamen	0.45 (0.09)	0.51 (0.11)	0.71 (0.15)	0.97 (0.27)	86.9	0.05 ^a , <0.0001 ^{bc}	0.011 ^d ; <0.0001 ^e , 0.001 ^f
Ratio R2*/vol G. Pallidus	2.13 (0.49)	2.72 (0.85)	(0.68)	4.33 (0.98)	86.54	0.002 ^a , <0.0001 ^{bc}	0.008 ^d ; <0.0001 ^e , 0.021 ^f

Values expressed as mean (S.D.) with the exception of gender. [†]*T*-student.

^aPreHD vs. Controls.

^bSS vs. Controls.

^cHD vs. Controls.

^dPreHD vs. SS.

^ePreHD vs. HD.

^fSS vs. HD.

and volume in the putamen ($P < 0.0001$; $r = -0.408$) and the globus pallidus ($P < 0.0001$; $r = -0.505$). The iron content in the putamen and globus pallidus correlated with the volume of all the three nuclei ($P < 0.0001$), but caudate iron content did not.

In cortical areas however, we found an inverse correlation between volume and iron content in the anterior cingulate ($P = 0.023$, 0.003 ; $r = -0.185$, -0.238) and a direct correlation in SMA-sensorimotor and temporo-occipital areas: left paracentral ($P = 0.019$, $r = 0.191$) and lateral occipital gyri ($P < 0.001$, $r = 0.316$); right transverse temporal ($P = 0.002$, $r = 0.254$) and pericalcarine gyri ($P = 0.006$, $r = 0.225$); and bilaterally within the entorhinal cortex (left: $P < 0.001$, $r = 0.292$; right: $P = 0.016$, $r = 0.292$), parahippocampus (left: $P = 0.001$, $r = 0.271$; right: $P < 0.001$, $r = 0.565$), fusiform gyrus (left: $P = 0.016$, $r = 0.179$; right: $P = 0.008$, $r = 0.216$), lingual gyrus (left: $P = 0.003$, $r = 0.242$; right: $P < 0.001$, $r = 0.390$), and cuneus ($P < 0.001$, left: $r = 0.338$; right: $r = 0.336$).

Correlations between basal ganglia volume and iron concentration and clinical measures

The iron content in the putamen ($P = 0.005$, $r = 0.318$) and the globus pallidus ($P = 0.008$, $r = 0.300$) was directly correlated with the HD-development index; while the volume of the three nuclei was inversely correlated with the HD-development: caudate ($P < 0.0001$, $r = -0.669$), putamen ($P < 0.0001$, $r = -0.806$) and globus pallidus ($P < 0.0001$, $r = -0.777$). Also, the iron content in the puta-

men ($P = 0.001$, $r = -0.375$) and volume in the caudate ($P < 0.0001$, $r = -0.426$) and globus pallidus ($P = 0.001$, $r = -0.373$) significantly correlated with the number of CAG repeats after correcting for the disease duration.

DISCUSSION

The aim of our study was to investigate iron distribution in the brain of presymptomatic and symptomatic Huntington's disease subjects and to determine the relationship between iron dysregulation and brain atrophy. To this end, we collected a large sample of Huntington's disease subjects all studied on the same MRI scanner and with identical scanning parameters. To our knowledge, this is the first time a study has detected differences in iron accumulation between presymptomatic subjects, symptomatic patients and subjects in a near-to-onset stage, that is, with the so-called "soft symptoms," and has yielded evidence of the relationship between volume loss and iron in GM regions.

With regard to the basal ganglia, four main findings emerge from our study. First, iron accumulates in the caudate nucleus, putamen and globus pallidus since presymptomatic stages. This iron load remains relatively stable in the caudate, while continues rising along disease stages in the putamen and globus pallidus. Second, a significant volume decrease in all the three nuclei since presymptomatic stages that decreases the more, the more advanced the disease stage. Third, basal ganglia volume (caudate, putamen and globus pallidus) is inversely related to iron

TABLE IV. Clinical characteristics of the sample (CAG repeats subgroups) including age and gender as covariates (‡)

	Controls (n=73)	First tertile ≤42 CAG (n=24)	Second tertile 43-44 CAG (n=24)	Third tertile ≥45 CAG (n=29)		Compared to controls X ² /F (P-value)	Differences between groups (P-value)
CLINICAL MEASURES							
Gender (M:F)	43:30	15:9	17:7	17:12	1.2	NS [†]	NS [†]
Age	44.52 (13)	50.92 (13)	47.13 (11)	37.31(9.6)	6.02	0.026 ^a , 0.008 ^{c++}	<0.0001 ^e , 0.004 ^{f++}
Disease duration (‡)	NA	4.88 (19.25)	0.38 (11.37)	3.86 (5.9)	22.11	NA	0.004 ^d , <0.0001 ^{eff++}
MMSE (‡)	NA	25.55 (4.01)	26.62 (3.34)	25.07 (3.8)	2.12	NA	NS
UHDRS Motor (‡)	NA	24.16 (14.9)	27 (24.05)	31.68 (21.35)	9.26	NA	0.0041 ^d , <0.0001 ^e , 0.014 ^{f++}
UHDRS Cognitive (‡)	NA	188.68 (80.6)	183.13 (86.32)	169.73 (51.16)	13.62	NA	0.0012 ^d , <0.0001 ^e , 0.004 ^{f++}
UHDRS Behavioral (‡)	NA	14 (10.28)	14.8 (9.75)	15.36 (9.8)	3.67	NA	0.009 ^{e++}
UHDRS Functional (‡)	NA	19.63 (7.11)	20.60 (5.5)	19.91 (5.85)	2.9	NA	0.02 ^{e++}
TFC (‡)	NA	10.05 (2.8)	10.55 (2.62)	8.58 (3)	5.11	NA	0.005 ^e , 0.023 ^{f++}
Independence scale (‡)	NA	88.75 (14)	89.09 (12.21)	77.08 (17.25)	5.6	NA	0.003 ^e , 0.018 ^{f++}
Disease burden (‡)	NA	312.33 (68.86)	339.27 (73.21)	539.17 (98.58)	33.62	NA	<0.0001 ^{e++}
MRI MEASURES							
TIV‡	1408291.6 (130506.64)	1347798 (12065.10)	1362179.7 (103437.52)	1332703.8 (130940.83)	4.44	0.046 ^b , 0.001 ^{c++}	NS ⁺⁺
Caudate R2* (‡)	18.93 (2.33)	19.89 (2.82)	18.96 (3.4)	18.37 (3.01)	96.55	NS	NS
Putamen R2* (‡)	24.91 (2.72)	27.83 (4.26)	27.09 (2.94)	26.46 (3.55)	81.5	0.002 ^a ; 0.009 ^b ; <0.0001 ^{c++}	NS
G. Pallidus R2* (‡)	34.14 (4.12)	39.61 (6.64)	39.03 (4.87)	40.33 (6.67)	90.47	<0.0001 ^{abct++}	0.039 ^e ; 0.031 ^{f++}
Caudate vol (‡)	3478.38 (401.76)	2615.33 (699.65)	2330.77 (533.8)	1934.27 (486.05)	0.42	<0.0001 ^{abct++}	0.012 ^d < 0.0001 ^{eff++}
Putamen vol (‡)	5637.22 (830.44)	3958.47 (1268.58)	3798.12 (1258.10)	3182.51 (834.62)	7.83	<0.0001 ^{abct++}	<0.0001 ^{eff++}
G. Pallidus vol (‡)	1638.36 (223.15)	1223.83 (337.21)	1134.18 (244.19)	967.46 (189.86)	17.93	<0.0001 ^{abct++}	0.039 ^d ; <0.0001 ^{eff++}
Caudate R2*/vol (‡)	0.55 (0.08)	0.81 (0.25)	0.84 (0.22)	1.01 (0.35)	45.16	<0.0001 ^{abct++}	<0.0001 ^{eff++}
Putamen R2*/vol (‡)	0.45 (0.094)	0.78 (0.3)	0.8 (0.29)	0.9 (0.31)	50.01	<0.0001 ^{abct++}	<0.0001 ^e ; 0.002 ^{f++}
G. Pallidus R2*/vol (‡)	2.13 (0.48)	3.50 (1.08)	3.61 (0.99)	4.34 (1.18)	71.98	<0.0001 ^{abct++}	<0.0001 ^{eff++}

Values expressed as mean (S.D.) with the exception of gender.

[†]Pearson's Chi-square.

⁺⁺T-student.

^aFirst tertile vs. Controls.

^bSecond tertile vs. Controls.

^cThird tertile vs. Controls.

^dFirst tertile vs. Second tertile.

^eFirst tertile vs. third tertile.

^fSecond tertile vs. third tertile.

content in the putamen and globus pallidus. Fourth, HD development and CAG repeats appear to influence the iron accumulation and the decrease in volume in these nuclei: the longer the disease duration and the higher the CAG repeats length, the higher the iron content and the smaller the volume, especially in the Putamen and globus pallidus.

With regard to the caudate, our results reveal iron accumulation and volume loss in this nucleus from as early as the presymptomatic stage. As the disease progresses, in the SS and HD stages, iron content remains relatively stable while volume decreases further. Two hypotheses in the literature may explain these phenomena: the first is related to the role of oligodendrocytes, while the second is related to that of neurons. The number of oligodendrocytes doubles in the early stages of disease [Gomez-Tortosa et al., 2001] probably due to their repair function in response to

progressive neuronal damage. As oligodendrocytes are cells with high iron content, an increased number of oligodendrocytes will raise the iron content in this nucleus [Bartzokis et al., 2007]. As regards the neuron hypothesis, previous mice models have shown that iron accumulates in neuronal cell bodies in gray matter areas [Smith et al., 2004]. Ferritin-bound iron distributes in the brain according to the different regional iron needs. Indeed, both dopamine and GABA, whose biosynthesis depends on iron availability, are the most frequent neurotransmitters in the striatum [Simmons et al., 2007; Zecca et al., 2004]. However, as iron transport and storage mechanism deteriorate, metabolically active iron may accumulate outside of ferritin and deteriorate cellular integrity by increasing oxidative stress [Zecca et al., 2004]. When neuronal loss becomes excessive, any attempt made by the

TABLE V. Stereotactic locations and Brodmann areas (BA) of significant differences between clinical groups and controls (VBM analysis, including age, gender and TIV as covariates)

Region (BA)	Cluster (<i>P</i> corrected)	Cluster size (mm ³)	MNI coordinates (<i>x, y, z</i>)	<i>T</i> -value ^a
Control > PreHD				
L Caudate	<0.0001	5284	−9 3 15	21.15
R Caudate			9 17 18	19.43
L Putamen	0.018	19	−24 5 9	5.71
R Precentral gyrus	<0.0001	8877	54 14 40	8.81
L Precentral gyrus (8)	<0.0001	157	−27 −37 75	6.39
R middle Frontal	<0.0001	619	57 35 25	8.73
L inferior Frontal (9)	<0.0001	530	−54 12 39	8.92
L Orbitofrontal (11)	0.001	274	−6 21 −30	5.63
L Premotor (6)	0.001	214	−32 0 64	6.49
L Postcentral gyrus (3)	<0.0001	423	−39 −42 66	8.10
R Postcentral gyrus (3)	<0.0001	440	40 −37 61	7.51
R Supramarginal (40)	<0.0001	221	43 −51 58	7.01
R precuneus (7)	0.001	267	10 −79 46	6.78
L Precuneus (7)	0.023	13	−8 −81 45	5.34
R middle Temporal	0.005	98	50 −34 −15	6.20
L inferior Temporal	<0.0001	448	−48 −22 −20	8.30
Control > SS				
L Caudate	<0.0001	1896	−9 9 13	15.83
L Putamen			−24 8 7	7.60
R Caudate	<0.0001	1814	9 17 10	14.00
R Premotor (6)	0.033	11	22 −9 73	4.95
R inferior Frontal (9)	0.010	59	54 14 40	5.55
R inferior Frontal (47)	0.045	8	45 18 −9	4.78
L inferior Frontal (9)	0.006	87	−54 12 37	6.26
L inferior Frontal (47)	0.019	18	−42 15 −9	5.96
L Postcentral gyrus (3)	0.008	73	−40 −43 64	5.41
L inferior Temporal	0.03	18	−48 −15 −26	4.97
Control > HD				
L Caudate	<0.0001	12667	−9 9 13	32.82
R Caudate			9 17 10	29.14
Posterior Cingulate	<0.0001	2250	14 −51 26	8.90
L inferior Frontal (47)	<0.0001	516	−42 15 −9	11.78
L superior Frontal (9)	0.001	154	−33 59 21	5.84
L middle Frontal	0.010	58	−46 48 21	4.91
R inferior Frontal	0.001	256	36 30 7	8.01
R Insula (13)	<0.0001	320	42 8 −9	7.91
R Parahippocampal gyrus	0.001	264	34 2 −27	9.05
R middle Temporal	0.001	249	50 −36 −14	7.16
L middle Temporal	<0.0001	878	−48 −25 −20	11.27
L middle Temporal (19)	<0.0001	483	−52 −78 16	7.18
R inferior Parietal (40)	<0.0001	799	40 −57 58	7.59
R Precuneus (7)	<0.0001	451	−6 −70 22	6.14
R Cuneus (17)	0.023	13	26 −81 28	5.21
L Supramarginal gyrus (40)	0.006	201	−42 −54 30	7.20
L Precuneus/Cuneus (7/17)	<0.001	483	−15 −52 37	7.18
			−10 −69 4	5.11
PreHD > SS				
L Putamen/Globus Pallidus	<0.0001	978	−24 9 −6	6.90
L Caudate			−19 5 21	6.58
R Putamen/ Globus Pallidus	<0.0001	266	21 11 −11	5.98
R Caudate	0.007	51	21 14 16	5.17
PreHD > HD				
L Putamen/Globus Pallidus	<0.0001	3591	−22 9 −6	12.66
L Caudate			−19 9 15	9.69
R Putamen	<0.0001	3062	22 11 −9	12.18
R Caudate			18 12 13	9.35
R Thalamus	0.016	23	10 −15 4	4.81

^aSignificance threshold $P < 0.05$ voxel-level corrected for multiple comparisons (FWE).

Figure 3.

Cortical areas. **(A)** Stereotactic locations of areas of significant $R2^*$ reduction (iron decrease) and volume loss in clinical groups compared to controls (age, gender and TIV as covariates; $P < 0.05$ FWE). **(B)** Cortical areas of significant differences between HD patients and control subjects. The filled triangles represent the iron decrease and the empty triangles the volume loss (Freesurfer parcellations; $P < 0.05$).

oligodendrocytes to prevent further neuronal loss fails, and as a result the number of oligodendrocytes and neurons decreases even further. These two phenomena thus presumably induce a global reduction in the nucleus volume and a decrease in iron content, as cells are no longer there, in which iron can accumulate. This would explain why iron levels increase in PreHD in comparison with controls, remain relatively stable in SS, and decreases in our HD patients according to the severe gray matter loss. However, if we calculate a ratio iron/volume (Supporting Information Fig. 1) we observe that iron in the caudate continues to increase in the different disease stages, though to a lesser extent than in the other nuclei.

Our results also reveal iron accumulation and volume loss in the putamen and globus pallidus, the deeper; the more advanced the disease stage. However, considering the apparently different disease progression in these nuclei compared with the caudate, we suggest that the caudate might be affected by a primary early impairment, whereas a secondary neurodegenerative process, might affect the putamen and globus pallidus. Giving support to this hypothesis is the strong correlation between caudate volume and iron content and volume in the putamen and globus pallidus ($P < 0.0001$). Iron accumulation in putamen and globus pallidus appears also to be influenced by the length of the CAG repeats and by the HD-development. Within the striatum, signs of pathology initially appear in the caudate nucleus and as the disease progresses, extend towards the putamen [Han et al., 2010]. The early iron accumulation and progressive volume loss in the caudate and putamen may be due to the selective, disease-induced vulnerability of GABAergic medium spiny neurons, largely and selectively localized in the striatum; and to the marked loss of D1 and D2 receptors in these type of neurons [Vonsattel, 2008]. As we said, iron is involved in the synthesis of GABA and dopamine and plays a major role in several physiological processes in neurons [Dumas et al., 2012, Simmons et al., 2007]. The deregulation of iron homeostasis may generate an excess amount of reactive iron, which would invade these cells. This invasion might damage neurons or perturb the cellular environment, rendering it more susceptible to toxins and activating pathogenic processes, that is, inflammation, factor release, morphological change, or apoptosis [Zecca et al., 2004]. As a consequence, there is a gradual progressive degeneration of the striatopallidal projections. Thus, iron accumulation in the globus pallidus may be a consequence of the progressive deafferentation of the striatopallidal pathway, as it has been suggested by previous studies [Douaud et al., 2009]. The medium spiny striatal neurons, project to the internal and external globus pallidus, thereby giving rise to the direct and indirect pathways. The staging system, based on neuropathological data [Vonsattel et al., 1985], describes a gradual atrophy of the caudate, putamen and external globus pallidus. The globus pallidus only displays atrophy in grades III and IV, with the external segment being affected to a far greater extent than the internal seg-

ment [Vonsattel, 2008]. Although tissue bulk decreases, neurons in the globus pallidus are relatively preserved. Thus, atrophy in this nucleus is apparently due to neuropil loss, and hence to degeneration of the striatal fiber connection and fiber passage, rather than to a loss of neurons [Vonsattel, 2008]. Second, as iron accumulates in normal aging in the putamen and globus pallidus [Cherubini et al., 2009; Langkammer et al., 2010], the increased iron level in those nuclei might be a manifestation of premature aging, that is, an acceleration of a physiological process.

Whether iron accumulation in the striatum and globus pallidus is a primary mechanism of tissue damage or a secondary effect of neuronal loss remains unclear. The correlation analysis performed in our study shows that the increase in iron content is inversely correlated to gray matter atrophy in the putamen and globus pallidus. This might be explained by: (1) the storage of iron in these nuclei in the remaining cells, which increases the iron/tissue ratio, as the R2*/volume ratio indeed seems to suggest; (2) the replacement of those nuclei, following neuronal loss, with other types of cells with a high iron content (i.e., oligodendrocytes) in an attempt to restore brain tissue; (3) a breakdown of the blood brain barrier, which allows more iron to access the brain [Kell, 2010]. A previous study did not find any correlation between the increase in iron levels and the decrease in volume in the striatum [Dumas et al., 2012]. Possible explanations of these diverse results are the different techniques used by Dumas et al.

Furthermore, as it has been suggested that CAG repeats may play a role in disease toxicity, we decided to investigate whether and, if so, how the CAG repeat length influences iron accumulation and volume loss in the basal ganglia. It is widely known that the number of CAG repeats may contribute to disease severity and to the time to disease onset [Langbehn et al., 2004]. A higher CAG repeat length has also been associated with faster clinical progression and faster striatal atrophy [Aylward et al., 2011a, 2011b; Rosenblatt et al., 2006]. Our results lend further support to previous findings by showing, first of all, that the higher the number of CAG repeats, the greater the decrease in volume. Also, volume loss in caudate and globus pallidus correlated with the number of CAG repeats after correcting for disease duration. Second, these results demonstrate for the first time that patients with higher number of CAG repeats (≥ 45) accumulate more iron in the globus pallidus than patients with a lower or medium number of CAG repeats. Also, iron content in the putamen correlates with the CAG repeats after correcting for disease duration. Third, iron content in the caudate and the putamen tend to decrease, even if not significantly in higher-CAG group in proportion to its volume decrease. In the higher-CAG group, the lower concentration of iron correlates with the greater atrophy in the caudate and putamen. As said before, when iron transport and storage mechanism deteriorate, metabolically active iron may accumulate outside of ferritin and deteriorate cellular integrity by increasing oxidative stress. A previous study

[Vymazal et al., 2007] found that the number of CAG repeats correlated with a decrease of ferritin in the caudate and an increase in the globus pallidus as measured by T2 relaxometry. They hypothesized that in the in globus pallidus iron was bound to ferritin, but not in the caudate where non-ferritin-bound iron exerted a toxic effect. Still, the differences in disease severity between the CAG groups could also be influencing these results.

Lastly, with regard to the cortex, our data show that: (a) iron levels in the cortex progressively decrease, that is, while they are normal in PreHD subjects, they are reduced in the parieto-occipital areas in SS subjects, and even more extensively reduced in HD patients, in whom the premotor and sensorimotor cortex and larger parieto-temporo-occipital regions are affected; (b) there is also a progressive loss in cortical volume, which starts in the frontoparietal areas (including premotor and sensorimotor areas) in presymptomatic subjects (PreHD and SS), and extends throughout the cortex, with the exception of the anterior frontal regions, in HD patients. This is the first study showing cortical iron disturbances in early stages of the disease. (c) Third, we found an inverse correlation between volume and iron content in the anterior cingulate and a direct correlation in paracentral (SMA-sensorimotor) and temporo-occipital areas.

Taken all together, these results suggest that iron has a secondary role in the pathophysiology of Huntington's disease in cortical gray matter as the regional volume loss in Pre-HD subjects occurs before any iron imbalance is detected. Furthermore, some of the cortical areas in which we found a correlation between volume and iron content are directly involved in the cortico-striatal circuits, such as SMA that is involved in the motor circuit; and the anterior cingulate, that participates in the limbic circuit. As, the striatum projects through the thalamus to these areas [Vonsattel, 2008]; the impairment of these cortical areas might be the consequence of the progressive loss of fibers (neurons starting from the striatum) projecting to the cortex via the thalamus. Another possible explanations for the decrease in iron availability in some cortical areas in concomitance with an increase in iron levels in the basal ganglia, will be the redistribution of iron to the basal ganglia according to a homeostatic mechanism [Rosas et al., 2012].

The only previous study that assessed iron concentrations in cortical structures [Rosas et al., 2012] reported an increase in iron levels in some cortical regions, that is, sensorimotor and parietal regions. Various reasons may explain the discrepancy between these results. First of all, Rosas et al. [2012] only found significant iron increases in cortical areas in patients with advanced disease (stage III), which were instead excluded from our study. By contrast, the only cortical areas in which they found significant differences in early disease stages (I-II) were occipital areas bilaterally and the right paracentral gyrus, in which iron decreases in patients compared to controls, consistently with our results. Second, we used a different acquisition technique from that adopted by Rosas et al. to measure

iron deposition as well as a different analytical approach. While the latter used a ROI study of some cortical regions based on previous neuropathological studies, we performed a whole-brain approach analysis not based on any *a priori* hypothesis.

In our study, we used R2* relaxometry as an indirect marker of iron deposition. This is a robust and established approach that has been applied in a number of clinical studies [Langkammer et al., 2012]. Our study does, however, have some limitations. The first limitation is that we do not have neuropathological confirmation of our findings. However, although we cannot definitively exclude all the potential sources of magnetic field variation, we believe that the increases in R2* observed in our study are related primarily to an increase in iron levels, in coherence with previous *ex-vivo* studies [Chen et al., 1993; Dexter et al., 1992; Schenck and Zimmerman, 2004; Simmons et al., 2007]. The increases in R2* caused by rising tissue iron levels are field-dependent, that is, the greater the field strength of the MRI instrument, the more marked the increase in R2* [Bartzokis et al., 2007]. As we used a high-field scanner, our results are likely to have been more sensitive to these iron increases. The second limitation of our study is that the R2* measures are also markedly sensitive to small changes in the amount of tissue water, which reduces the R2* and may mask changes in iron content. These decreases in R2* are field-independent, which means that we cannot improve acquisition by increasing the scanner field. Also, some cortical regions, such as the orbitofrontal areas and the insula, are very sensitive to artifacts in the MRI signal owing to its location. For this reason, two experienced raters visually assessed all the brain masks during the brain extraction to ensure that artifacts were excluded from the analysis. Also, the caudate nucleus is not an easy structure to be automatically segmented owing to its proximity to the ventricles. We thus performed a ROI analysis by means of two different techniques and subcortical segmentations were visually controlled to ensure the proper inclusion of the whole nucleus. Finally, the HD-development index, is the result of the combination of two different scales that have different level of accuracy, because one is a predicted 'time to onset' estimated by a formula, while the other is the result of the interview with the patient and family members and results on the clinical scales.

CONCLUSIONS

In conclusion, these data suggest that iron alterations occur early in the Huntington's disease process and may play a role in the selective striatal degeneration underlying Huntington's disease. We observed a progressive increase in iron levels and a decrease in volume, starting in the caudate and subsequently affecting the putamen and globus pallidus, which manifest themselves from as early as the presymptomatic stages. Iron accumulation and

volume loss worsen along disease stages and in proportion to the number of CAG repeats. Besides, cortical areas display a reduction in iron content and volume loss predominantly in the posterior areas. Furthermore, there is an inverse correlation between volume and iron levels in putamen, globus pallidus and the anterior cingulate; and a direct correlation between volume and iron content in cortical structures, that is, SMA-sensorimotor and temporo-occipital areas. Taken all together, it seems iron homeostasis is affected in the disease. However, there appear to be differences in the role played by iron in basal ganglia and in cortical structures, whereas gray matter volume loss progresses in the same way in all the structures. The marked variability of the relaxation rates and their complex relationship with gray matter atrophy suggest that the two techniques might be sensitive to complementary characteristics of brain tissue, and may thus be able to shed further light on the neurodegenerative processes underlying Huntington's disease. Longitudinal studies are warranted to confirm the findings of our study and gain an even deeper understanding of the role of iron in the pathogenesis of Huntington's disease.

ACKNOWLEDGMENTS

This study was possible thanks to the collaboration of HD patients and families, members of Lega Italiana Ricerca Huntington e malattie correlate onlus (www.lirh.it). We also thank Lewis Baker for editing the English in the manuscript.

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