Plasma 24S-hydroxycholesterol and caudate MRI in pre-manifest and early Huntington’s disease

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Huntington’s disease (HD) is a hereditary neurodegenerative disorder for which biological indicators of disease progression, or disease stage, would be especially important for therapeutic trials. 24S-hydroxycholesterol (24OHC) is a brain-generated cholesterol metabolite which has been associated with neurodegeneration, and alterations of cholesterol metabolism in murine HD models and patients’ tissues have been recently identified. On these grounds, and with the aim of identifying putative biomarkers in HD, we studied cholesterol metabolism through the analysis in vivo of plasma 24OHC and cholesterol in two independent cohorts of controls and patients of Italian and British origin. We analysed a total of 62 controls, 96 HD symptomatic patients at different disease stages (stage 1–3), and 33 HD gene-positive pre-manifest subjects (pre-manifest HD (pre-HD)). Cholesterol and 24OHC plasma levels were comparable in both the British and Italian subjects, and were not influenced by fasting or post-meal status. Cholesterol levels did not show differences between controls, pre-HD subjects and HD patients. In contrast, the plasma levels of 24OHC were significantly higher in controls than in HD patients at all disease stages (P < 0.001). Interestingly, in pre-HD subjects plasma 24OHC concentrations were similar to those of controls, and thus significantly greater than those of HD patients at any disease stage (P < 0.001). As expected, significant differences in caudate volumes between stage 1–2 HD patients and pre-HD subjects, and pre-HD subjects and controls were found. The pre-HD cohort of subjects was heterogeneous as to 24OHC levels, since subjects closer to predicted development of motor signs of disease had lower 24OHC levels than those far from onset. Our data indicate that the brain-generated cholesterol metabolite 24OHC measured in plasma was significantly depleted in HD patients at any disease stage, and it could discriminate pre-manifest subjects from patients with overt motor disease. However, 24OHC levels failed to mark further disease progression in patients with manifest HD. Overall, we demonstrate that 24OHC levels parallel the large decrease in caudate volumes observed in gene-positive subjects from pre-manifest to HD stage I, thus reflecting a critical phase characterized by neuronal loss. We conclude that that 24OHC levels complement MRI morphometry as a valuable tool to follow neurodegenerative changes in the early stages of Huntington disease.

Keywords: gas chromatography-mass spectrometry; oxysterol; biomarker; 24S-hydroxycholesterol; caudate volume; MRI

Abbreviations: HD = Huntington’s disease; 24OHC = 24S-hydroxycholesterol; pre-HD = pre-manifest HD;
CAG = Cytosine – Adenine – Guanine


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Introduction

Huntington’s disease (HD) is an inherited neurodegenerative disorder caused by a dominant glutamine expansion within the N-terminus of the huntingtin protein (Huntington’s Disease Collaborative Research Group, 1993). The HD pathogenesis is complex and heterogeneous, although there is a general consensus on the concept that the mutant HD gene confers a toxic gain of function to the corresponding protein. A persuasive line of evidence for this idea (Walker, 2007) comes from the eight other known human genetic disorders with expanded polyglutamine repeats sharing with HD progressive neurodegeneration and the very same genetic mutation (Taroni and Di Donato, 2004). In HD, the protein context plays an important role in orienting neurodegeneration, as the huntingtin protein, and particularly its truncated products, are highly toxic because they tend to auto-aggregate and to promote aberrant protein–protein interactions in several sub-cellular districts including the nucleus (Graham et al., 2006). In addition, loss of individual beneficial activities of normal huntingtin in brain cells may contribute to disease pathogenesis (Cattaneo et al., 2005). These multifaceted molecular events ultimately lead to neuronal dysfunction and death, probably through a combination of transcriptional repression, proteasome impairment, oxidative injury and mitochondrial dysfunction.

Notably, the loss in brain mass is quite substantial in HD: in the 15–20 years of the disease course from the onset of symptoms to death, the brain weight declines in patients from 1350 g to <1100 g (Vonsattel and Di Figlia, 1998). The clinical associates of such dramatic neurodegeneration, which mostly occurs in the striatum and cortex and eventually spreads throughout the brain (Rosas et al., 2003), are progressive motor deterioration—mainly chorea and dystonia—psychiatric and behavioural abnormalities, which may precede or accompany the motor onset, and cognitive decline (Marder et al., 2000). Neuropathological findings in HD are mirrored by evidence of progressive atrophy of the striatum on MRI scans (Aylward et al., 2004). These nuclei appear particularly sensitive to the toxic effects of mutant huntingtin, and degenerative changes, can be seen not only in the first stages of the disease (HD stage 1, see below), but also in gene-positive pre-manifest subjects who are close to the estimated age-at-onset (Aylward, 2007). Accordingly, quantitative MRI scans of the caudate in HD patients have been recently considered as possible valid measures of disease progression and putative outcome measures in clinical trials, as caudate volumes were shown to positively correlate with the clinical decline as measured by scores (Aylward, 2007). Though encouraging in view of prospective therapeutic trials, because of evidence of more widespread degeneration in early HD (Rosas et al., 2003), these data require further refinement before being considered a unique biomarker of neurodegeneration, particularly over a relatively short period of time. Thus, clinical investigators face a problem as to the objective evaluation of therapeutics, as measurement of Cytosine–Adenine–Guanine (CAG) repeat number is clearly not predictive of the response to treatment in clinical trials, which have to rely on relatively insensitive clinical measures. In this context, it appears imperative to develop tools which might facilitate the assessment of disease state and the efficacy of new therapeutics, particularly those that may help to predict the early stages of disease and the long period that precedes the onset of overt motor signs. In fact, an effective therapeutic approach is most likely to be successful in pre-manifest subjects who have not yet developed irreversible neurodegeneration. In this context, some of us (S. J. T. and E. J. W.) have identified changes in the plasma proteomic profile in HD patients suggesting that signals of inflammatory response are detectable in the periphery and may follow disease progression (Dalrymple et al., 2007).

We present here an additional approach based on the in vivo study of cholesterol metabolism in plasma from HD patients, combined with MRI morphometry of the brain. This approach stems from two independent lines of investigation. First, we considered the general problem of cholesterol metabolism in brain taking into account the following: (i) cholesterol represents ~2% of the wet weight of the brain (Björkhem and Meaney, 2004); (ii) most de novo biosynthesis happens during brain development, whereas de novo cholesterol biosynthesis in the adult brain is limited (Dietschy and Turley 2004); (iii) cholesterol homeostasis is essential for proper brain function (Pfrieger, 2003), and a constant equilibrium between synthesis and degradation is maintained through the oxidation of cholesterol to 24S-hydroxycholesterol (24OHC) catalysed by the cholesterol-24-hydroxylase CYP46 (Lund et al., 1999), a neuron-specific enzyme particularly enriched in the striatum and the cortex, that generates a metabolite able to cross the blood–brain barrier (BBB); (iv) knocking out cholesterol 24-hydroxylase causes 40% reduction in de novo cholesterol synthesis in the brain, despite steady state levels of cholesterol being similar to controls, suggesting a strict control of total cholesterol content in the brain (Lund et al., 2003); (v) in the human adult brain cholesterol turnover gives rise to a daily flux of about 7 mg of 24OHC from the brain into the circulation (Lütjohann et al., 1996), so that 24OHC levels in plasma are taken as an index of brain cholesterol elimination. Accordingly, plasma 24OHC has been utilized as a putative marker of brain cholesterol turnover in neurodegenerative diseases, including Alzheimer disease and multiple sclerosis (Teunissen et al., 2005; Björkhem et al., 2006b). Overall, it has been proposed that the levels of 24OHC in the circulation reflect the number of metabolically active neurones in the brain, and thus the volume of the brain grey matter (Björkhem, 2006a).

In addition, there appears to be disturbed cholesterol biosynthesis in HD as key genes involved in cholesterol biosynthetic pathway were reduced in inducible mutant
huntingtin cell lines (Sipione et al., 2002), in striatum and cortex from R6/2 huntingtin-fragment mice and in HD brain and fibroblasts (Valenza et al., 2005). Additional studies showed that cholesterol synthesis in the brain was significantly reduced in the R6/2 mice and in the yeast artificial chromosome (YAC128) mice, as indicated by reduced levels of the cholesterol precursors lanosterol and lathosterol, and by decreased activity of 3-hydroxy-methylglutaryl-CoA-Reductase (Valenza et al., 2007a, b). Notably, 24OHC levels in plasma of the 10-month-old YAC128 mice were also decreased (Valenza et al., 2007b).

Overall, these data suggested that 24OHC might be a valuable peripheral biomarker to investigate disease severity and the neurodegenerative process in HD. Accordingly, we performed an in vivo analysis of cholesterol metabolism in HD subjects. In plasma from 62 controls, 96 HD patients and 33 pre-manifest HD (pre-HD) subjects, we sought to measure the levels of cholesterol, and the levels of the neuronal-generated cholesterol catabolite 24OHC. Through MRI morphometric analysis, we also evaluated caudate volumes in controls, pre-manifest and HD patients, and investigated associations between these parameters and the 24OHC plasma levels.

**Material and Methods**

**Patients**

Italian HD subjects were recruited for study between April, 2005 and May, 2007. A total of 72 HD mutation-positive individuals and 42 controls subjects were enrolled for the study at the Istituto Neurologico Carlo Besta, Milan, Italy. Patients’ and controls’ blood samples were always withdrawn after an overnight fast. British HD subjects were recruited between October, 2005 and July, 2007. Blood was collected from 77 non-fasting individuals, including 57 HD mutation-positive subjects and 20 controls, between 1 and 4 p.m.

Italian subjects were studied as inpatients admitted to the Besta Institute for full neurological, MRI and metabolic evaluation, and blood was collected after an overnight fast in agreement with the current procedures for studies on lipid metabolism (Cohn et al., 1988).

British subjects were studied as outpatients, and blood was taken in the early afternoon to coincide with outpatient visits, since subjects could not be expected to remain fasting until this time. None of the Italian and British controls or patients had primary abnormalities of cholesterol metabolism or was under statin treatment. Controls and patients within the two cohorts of Italian and British subjects followed similar dietary habits.

To investigate the possible effect of the fasting and post-meal state on cholesterol metabolism, we analysed 20 normal subjects (10 Italian and 10 British) for relevant metabolites before and after an identical light meal.

All eligible participants received verbal and written information about the study, and signed an informed consent form, according to the Declaration of Helsinki. For symptomatic HD patients with severe cognitive impairment, the informed consent was obtained from his/her relative. The study protocol was approved by the Ethics Committees of the C. Besta Neurological Institute and the UCL/UCLH Joint Ethics Committee. All the patients were evaluated by a standard neurological examination. Detailed family history, age of onset of symptoms, medications and other relevant clinical information were collected.

All subjects were considered for MRI scanning. Patients were selected according to willingness to undergo MRI scanning, absence of contraindications such as claustrophobia and clinical suitability (likelihood of remaining motionless in the scanner for the duration of the scan).

Disease-specific inclusion criteria were the following:

(a) Symptomatic HD patients. Ninety-six HD patients positive for the molecular test for the presence of a CAG triplet repeat number >35 in the Huntington gene and with manifest clinical signs and symptoms of the disease. Clinical assessment of motor symptoms and total functional capacity (TFC) were determined using the Unified Huntington’s Disease Rating Scale (UHDRS) (Huntington Study Group, 1996). Disease stage was determined according to Marder’s specifications (Marder et al., 2000). The age at onset was considered the time when motor symptoms were first noticed. Patients with only psychiatric manifestations were excluded from the study.

(b) The pre-HD individuals. Thirty-three individuals were selected among those who decided to undergo the programme for pre-manifest genetic testing according to the International Huntington’s Association (IHA) and World Federation of Neurology (WFN) guidelines (IHA and WFN Research Group on Huntington’s Chorea, 1994). We enrolled people with more than 35 CAG triplet repeats, having no clinical symptoms or signs of the disease and a UHDRS motor diagnostic confidence score of <4. For each pre-HD individual the estimated probability of onset within the next 5 years was calculated on the basis of the CAG repeat number and his/her present age following the model of Langbehn (Langbehn et al., 2004).

(c) Control individuals. Controls from Italy were 22 spouses and 20 normal volunteers from the Lombardy Region who had normal fasting values for cholesterol, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), glucose and triglycerides. Controls from the UK were 15 partners/spouses and 5 mutation-negative family members with normal cholesterol plasma values.

Patients and controls aged <18 years, or having known major medical conditions in addition to the primary genetic disorder, were excluded.

**Blood sampling**

Blood for cholesterol metabolite analysis in the Italian cohort was drawn between 8 and 10 a.m. after overnight fast. Plasma was obtained by centrifugation at 2100 r.p.m., and aliquots of 1 ml were immediately frozen at minus 70°C, until extraction and analysis. Blood for cholesterol metabolite analysis in the British cohort was taken between 1 and 4 p.m., and plasma was obtained as previously described (Dalrymple et al., 2007).

**Genetic assessment**

The DNA for genetic diagnosis was extracted from venous lymphocytes. Genetic testing for HD was performed in the laboratory of Biochemistry and Genetics of the Neurological Institute C. Besta, and in the Clinical Neurogenetics Laboratory of the National Hospital for Neurology and Neurosurgery, according to published methods (Gellera et al., 1996).
MRI acquisition parameters

Magnetic resonance imaging (MRI) was performed in a subset of 39 subjects from the Italian cohort, and in a subset of 34 subjects from the British cohort. All subjects were scanned with a 1.5 T MR system, 100% of Italian subjects and 96% of British subjects within 24 hours of blood sampling. Volumetric TI-weighted images for the Italian subjects were acquired with a Siemens Magnetom Avanto (Erlangen, Germany) by means of a magnetization-prepared gradient-echo sequence (MPRAGE) (160 sagittal sections, TR = 1640 ms, TE = 2.0 ms, TI = 552 ms, isotropic voxel size 1 x 1 x 1 mm; flip angle 12°).

For the British subjects an equivalent sequence for GE scanner was used (http://bitc.bme.emory.edu/acronyms.html): an IR prepared FAST spoiled Grass sequence (3D-FSPGR) (24 cm x 75% field of view, 256 x 256 matrix; 124 contiguous 1.5-mm thick coronal slices. In-plane pixel dimensions: 0.9375 x 0.9375 mm. TR = 13 ms; TE = 5.2 ms; flip angle = 13°; TI = 650 ms; receiver bandwidth = 16 kHz; NEX = 1).

MRI morphometric analysis

All caudate volumes were measured on T1-weighted 3D MR by means of a toolbox for automatic structure segmentation of MRI images, the Individual Brain Atlases using Statistical Parametric Mapping Software (IBASPM) developed in MATLAB (http://www.mathworks.com). The processing of the T1 images consists of normalization to Montreal Neurological Institute (MNI) space to obtain the spatial transformation matrix and the labelling of each voxel on the basis of MNI anatomical atlas (Tzourio-Mazoyer et al., 2002). The resulted structures of interest were extracted using our Matlab script and then corrected with FSL View Software (http://www.fmrib.ox.ac.uk) considering some standard features. The most caudal slice of basal ganglia was the slice in which the caudate and putamen were clearly separated by the internal capsule. The caudate was bordered laterally by the internal capsule and medially by the lateral ventricle. The measure progressed until the body of the caudate was no longer visible. Both the body and the tail of the caudate were included in the measurement. The borders of the putamen were defined laterally by the internal capsule. At more caudal levels, the medial borders of the putamen were defined by the globus pallidus; at more cranial levels, the medial borders were defined by the internal capsule. The correction was made in all the three planes to make certain that no tissue was omitted. The segmentation of caudates in the British and Italian subjects, and the corresponding volumetric data have been calculated by the same method and by a unique rater (M.L.M. in Milano). Caudate volumes are presented both as absolute volumes, and as a ratio to Total Intracranial Volume (TIV), in order to adjust for differences in head size. (Whitwell et al., 2001) (Table 3 and Table S3).

Isotope dilution mass spectrometry analysis

To a screw-capped vial sealed with a Teflon-lined septum 250 μl of plasma were added together with 100 ng of [2H4] 24OHC as Internal Standard. To prevent autoxidation 25 μl of butylated hydroxytoluene (5 g/l) and 25 μl of EDTA (10 g/l) were added to each vial and argon was flushed through the vial to remove air. Alkaline hydrolysis was then allowed to proceed at room temperature (22°C) with magnetic stirring for 1 h in the presence of ethanolic 1 M potassium hydroxide solution. After hydrolysis the sterols were extracted twice with 5 ml of cyclohexane. The organic solvents were evaporated under a gentle stream of argon and converted intotrimethylsilyl ethers (pyridine:hexamethyldisilazane:trimethylchlorosilane 3:2:1 v/v/v). Gas chromatography-mass spectrometry (GC-MS) analysis was performed on an Agilent Technologies HP 5890 series II combined with a 5972 mass selective detector. The GC was equipped with a DB-XLB (30 m x 0.25 mm id x 0.25 μm film; J&W, Palo Alto, CA, USA) and injection was performed in the split less mode using helium (1 ml/min) as a carrier gas. The temperature programme was as follows: initial temperature of 180°C kept for 1 min, followed by a rise of 20°C/min up to 260°C and immediate rise by 10°C/min up to the end temperature of 280°C, kept for 15 min. The mass spectrometer was operated in the selected ion-monitoring mode. Neutral sterols were monitored as their TMSi derivates in the selected-ion-monitoring mode using the following masses: 24OHC at m/z 413 [M+-OTMSi-CH(CH3)2], [2H4]24-OHC at m/z 416 [M+-OTMSi-CD(CH3)2], lanosterol at m/z 498. Peak integration was performed manually, and sterols were quantified from selected-ion-monitoring analyses against internal standards using standard curves for the listed sterols. Identity of sterols was proven by comparison with the full-scan mass spectra of authentic compounds. Additional qualifier (characteristic fragment ions) ions were used for structural identification (Dzeletovic et al., 1995; Leoni et al., 2002). The analysis of lathosterol was performed by isotope dilution mass spectrometry: 50 μl of plasma were mixed with 100 ng of 5α-Cholest-7-en-3b-ol-1,2,5a,6a-d4 (2H4-lathosterol, CDN isotopes) and then the procedure was similar to the one described above. Lathosterol was monitored as its TMSi derivate in the selected-ion monitoring mode using the following mass: [H4]-lathosterol at m/z 462 (M+-OTMSi, lanosterol at m/z 498). Peak integration was performed manually, and sterols were quantified from selected-ion-monitoring analyses against internal standards using standard curves for the listed sterols. Identity of sterols was proven by comparison with the full-scan mass spectra of authentic compounds. Additional qualifier (characteristic fragment ions) ions were used for structural identification (Dzeletovic et al., 1995; Leoni et al., 2002).

Cholesterol was analysed by standard spectrophotometric method (Roche Diagnostic-Applied Science, Monza, Italy).

To test for variations due to fasting or post-meal levels in cholesterol metabolites, 10 Italian Hospital workers and 10 healthy British control subjects were tested before and 2 h after a standard light meal.

Statistical analysis

Continuous data were inspected and tested to determine whether distributions were normal by Kolmogorov–Smirnov normality test, and compared using Kruskal–Wallis test for non-parametric data, or Analysis of Variance (ANOVA) for parametric data. Comparison between two groups was performed with two-tailed Student’s t-test. Correlations were computed using Pearson coefficients. The ANOVA was used for comparison of plasma 24OHC in the different disease groups (HD, pre-HD and control subjects). All analyses were performed with Sigmastat 3.01 (SigmaAldrich, St Louis, MO, USA).

Results

To examine whether the fasting or post-meal condition might introduce possible bias in cholesterol metabolite analysis we analysed, before and after a light meal, 20 healthy individuals (10 Italian and 10 British controls) for consistent metabolic parameters: total and fractionated (HDL and LDL) cholesterol, triglycerides, 24OHC and
glucose did not show significant changes; however, lathosterol and lanosterol levels were significantly lower after meal in the British controls, and lathosterol was significantly lower after meal in the Italian controls. (Table S1). From this ad hoc analysis of 20 healthy controls we inferred that cholesterol biosynthesis may be inhibited or down-regulated after a meal (Table S1). We therefore focused our study on the plasma levels of cholesterol and 24OHC.

Table 1 shows age, CAG repeat number, UHDRS and TFC for each group of individuals considered. Control age did not significantly differ from HD patient age. However, as expected, age for pre-HD subjects differed from that of controls and from HD patients at any disease stage. The possible confounding effect of age differences was considered by selecting a subgroup of age-matched controls for direct comparison with pre-HD subjects, and no differences were found for cholesterol ($P = 0.93$, student $t$-test), 24OHC levels ($P = 0.58$, student $t$-test), and 24OHC/cholesterol ratio ($P = 0.6$, student $t$-test). No significant differences were found about age between controls and HD (stage 1–3) patients (ANOVA).

Plasma levels of cholesterol, 24OHC and 24OHC/cholesterol ratio in controls, pre-HD subjects and HD patients of both Italian and British origin are reported in Table 1. The HD patients have been divided according to the stage from early (HD1), to moderate (HD2) and to advanced disease (HD3). Plasma cholesterol levels did not differ between controls and gene-positive subjects. The levels of 24OHC and 24OHC/cholesterol ratio in HD patients at any disease stage were significantly lower than in controls in both cohorts, with the exception of the small group of British HD2 patients (Table 1). In pre-manifest subjects levels of 24OHC were similar to those of controls, and thus significantly higher than those of HD patients. A comparative analysis of cholesterol metabolites in the Italian and the British cohort of controls, pre-manifest and HD1–HD3 patients showed that plasma values for 24OHC, cholesterol and 24OHC/cholesterol ratio did not statistically differ (Table S2). We therefore merged the data from the two populations to maximize the statistical power of the study. Combined analysis (Table 2), showed a statistically significant difference in the plasma concentrations

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic, clinical and biochemical characteristics of the Controls, pre-manifesting subjects and HD patients</th>
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<tbody>
<tr>
<td></td>
<td>Italian subjects</td>
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<tr>
<td></td>
<td>Controls (n = 42)  Pre-HD (n = 18)  HD1 (n = 31)  HD2 (n = 12)  HD3 (n = 11)  ANOVA P</td>
</tr>
<tr>
<td>Female/Male</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>26.0 ± 14  32.7 ± 8.8  45.7 ± 9.1  52.4 ± 9.1  57.2 ± 13  &lt;0.001</td>
</tr>
<tr>
<td>Expanded allele (CAG)</td>
<td>-  44 (39–47)  45 (40–52)  45 (40–55)  44 (39–48)  -</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>-  5.3 ± 4.7  5.7 ± 2.5  9.9 ± 5.2  0.028</td>
</tr>
<tr>
<td>24OHC</td>
<td>57.9 ± 59  578 ± 78  45.8 ± 79$^<em>$  44.6 ± 15$^</em>$  42.3 ± 58$^*$  &lt;0.001</td>
</tr>
<tr>
<td>Chol</td>
<td>2.02 ± 2.1  1.89 ± 0.2  2.04 ± 0.3  2.00 ± 0.4  1.82 ± 0.3  0.235</td>
</tr>
<tr>
<td>24OHC/cholesterol ratio</td>
<td>29.3 ± 6.2  30.7 ± 39  22.9 ± 4.3$^<em>$  22.3 ± 6.1$^</em>$  23.5 ± 3.7$^*$  &lt;0.001</td>
</tr>
</tbody>
</table>

24OHC = 24S-hydroxycholesterol; chol = cholesterol. Results are presented as Mean ± SD. Statistics: ANOVA (statistical significance for $P < 0.05$). $^*$P < 0.001, by all pairwise multiple comparison procedures, with Holm–Sidak method for HD (stage I–3) compared with controls and pre-manifesting HD subjects. $^{**}$P < 0.05, by all pairwise multiple comparison procedures, with Holm–Sidak method for HD (stage I–3) compared with Controls and pre-manifesting HD subjects (see also supplementary Table S2 for the comparison between UK and Italian subjects).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Plasma levels of 24OHC and cholesterol of controls, pre-manifesting and manifesting HD patients of the two cohorts unified (Italian and UK cohort)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Controls (n = 67)  Pre-HD (n = 33)  HD1 (n = 46)  HD2 (n = 20)  HD3 (n = 30)</td>
</tr>
<tr>
<td>24OHC</td>
<td>575 ± 10.8  569 ± 8.4  46.3 ± 9.2$^{ab}$  45.8 ± 14.3$^{ab}$  46.0 ± 10.4$^{ab}$</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>198 ± 0.36  1.87 ± 0.23  2.02 ± 0.33  1.90 ± 0.41  1.93 ± 0.36</td>
</tr>
<tr>
<td>24OHC/cholesterol ratio</td>
<td>299 ± 711  308 ± 5.48  23.2 ± 4.30$^{ab}$  24.7 ± 798$^{ab}$  24.0 ± 4.51$^{ab}$</td>
</tr>
</tbody>
</table>

Results are presented as Mean ± SD. ANOVA with Holm–Sidak multiple comparison method. Compared with controls: $^a$P < 0.001. Compared with pre-HD: $^b$P < 0.001.
of 24OHC between controls and HD patients \((P < 0.001)\). Plasma 24OHC levels in pre-HD subjects were not significantly different from controls, but they were significantly higher than those observed in HD1–3 patients \((P < 0.001, \text{Table 2 and Fig. 1A})\). No significant differences were seen between groups of patients.

To evaluate the degree of neurodegenerative changes in HD brain we compared caudate volumes and the caudate/TIV ratio in a group of subjects (British \(n = 54\); Italian \(n = 39\), Table S3).

Caudate volumes were significantly lower in HD1 and HD2 patients than in controls and in pre-manifest subjects \((P < 0.001)\). Caudate volumes in HD1 and HD2 patients did not significantly differ. Pre-manifest caudate volumes were significantly lower than in controls (Table 3). Similar statistical findings were found for the caudate/TIV ratio (Table 3 and Fig. 1B). Comparable results were found also for the Italian and UK cohort separately analysed: caudate and caudate/TIV ratios were significantly lower compared with control subjects \((P < 0.001)\); pre-manifest caudate and caudate/TIV ratios resulted significantly lower compared with control subjects \((P < 0.01)\), (ANOVA, with Holm–Sidak method for multiple comparison).

The 24OHC levels (Fig. 1A) were significantly lower in HD1–HD2 patients compared to controls and pre-manifest subjects, but they did not differ between pre-manifest and control subjects. We reasoned that these inconsistencies of the 24OHC levels might be associated with some heterogeneity in the pre-manifest subjects in terms of their distance from motor onset. Hence, we investigated in the pre-manifest subjects putative relationships between 24OHC and caudate volumes and the probability of developing overt motor features. We found that caudate volumes in pre-HD did not correlate significantly with the probability of motor onset \((r = 0.374, P = 0.06, \text{Fig. 2A})\). However, plasma 24OHC in these subjects showed a significant inverse correlation with the probability of motor onset \((r = 0.41, P = 0.02, \text{Fig. 2B})\). No significant correlation was found between 24OHC and caudate volume \((r = 0.22, P = 0.29, \text{Fig. 2C})\). Since caudate volumes and 24OHC levels (Fig. 1A and B) showed a similar decremental pattern from pre-manifest to HD stage 1, we examined the relationships between plasma 24OHC levels and neurodegenerative changes, and plotted values for plasma 24OHC and caudate volumes in 26 pre-manifest and 23 HD1 patients. Statistical analysis showed that the decrement in plasma 24OHC levels was significantly and positively correlated with caudate volume losses \((r = 0.44, P = 0.0015, \text{Fig. 3})\).

**Table 3** Comparison of volumetric MRI analyses in controls, pre-HD, and HD patients at different disease stages

<table>
<thead>
<tr>
<th></th>
<th>Controls ((n = 31)) (UK/ITALY, 16/15)</th>
<th>Pre-HD ((n = 26)) (UK/ITALY, 15/11)</th>
<th>HD 1 ((n = 23)) (UK/ITALY, 15/8)</th>
<th>HD 2 ((n = 13)) (UK/ITALY, 8/5)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudate (mm(^3))</td>
<td>7332 ± 769.4</td>
<td>6723 ± 974.3a</td>
<td>4074 ± 860.8b,c</td>
<td>3731 ± 899.4b,c</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TIV (cm(^3))</td>
<td>1462 ± 1491</td>
<td>1517 ± 155a</td>
<td>1495 ± 100.7b</td>
<td>1423 ± 168.3b</td>
<td>0.22l</td>
</tr>
<tr>
<td>Caudate/TIV ratio (mm(^3)/cm(^3))</td>
<td>5.05 ± 0.61</td>
<td>4.44 ± 0.55b</td>
<td>2.73 ± 0.54b,c</td>
<td>2.64 ± 0.67b,c</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results presented as Mean ± SD. Statistic: ANOVA with Holm–Sidak method for multiple comparison. Compared with controls: \(^aP < 0.01\); \(^bP < 0.001\). Compared with pre-HD: \(^cP < 0.001\).
Discussion

We report here novel data on in vivo plasma cholesterol metabolism and caudate volume loss in a large cohort of pre-manifest and manifest HD derived from two independent populations. The levels of cholesterol and its brain-derived catabolite 24OHC were consistent in the plasma of controls and HD-gene-positive subjects from the two different European populations analysed. Pre-HD subjects and HD patients had normal levels of plasma cholesterol.

The brain is the most cholesterol-rich organ in the body (Björkhem and Meaney, 2004), and the BBB efficiently prevents cholesterol uptake from the circulation into the brain, so that de novo synthesis is responsible for almost all cholesterol present in this organ. As a consequence of the BBB, there is a highly efficient apolipoprotein-dependent recycling of cholesterol in the brain, with minimal losses (Björkhem et al., 2006a). Thus brain cholesterol content is totally dependent on endogenous cholesterol synthesis as well as on endogenous catabolism.

Notably, in plasma collected from HD patients at any disease stage we found significantly reduced levels of the neuronal-specific 24OHC. The concentrations of 24OHC were similar in pre-HD subjects and in controls, but significantly greater in pre-HD subjects than in HD patients at any disease stage (P < 0.001). Separate analysis of the two cohorts in Table 1 shows that 24OHC levels in the British subgroup of HD2 patients were not significantly different from those of their controls. We attribute this inconsistency to the small size of this group of patients. An additional explanation is that feeding might slightly influence the distribution of lipids carried by the HDL and LDL fractions, leading to greater variability in the distribution of the 24OHCh levels.
Because levels of 24OHC in the circulation are considered to reflect the number of metabolically active neurones in the brain, and thus the volume of the brain grey matter, we examined the association between the degree of brain atrophy and 24OHC levels in the pre-HD subjects and HD patients through MRI volumetric analyses of the caudate in subjects collectively obtained from British and Italian groups. This analysis provided evidence for a significant difference \( P < 0.001 \) in caudate and TIV-adjusted caudate volumes between pre-HD and HD patients (Table 3). 24OHC levels in plasma showed a significant reduction of this metabolite in the HD group (Table 2).

We reasoned that our pre-manifest group of subjects might be heterogeneous, and we found that pre-manifest subjects with a low probability of becoming symptomatic within 5 years had higher levels of 24OHC, similar to those of controls, whereas the few subjects closer to the predicted clinical onset had lower 24OHC levels (Fig. 2B). The correlation between 24OHC and caudate volumes in pre-manifest subjects, however, was not statistically significant. We interpret these data as a consequence of the fact that the majority of our pre-manifest subjects were far from predicted motor onset. (Fig. 2C).

In this study we also found that 24OHC levels, though altered in early HD, did not seem to follow disease progression based on correlations with clinical HD staging from the functional TFC values (Table 2 and Fig. 1A). We do not offer a definitive explanation for this finding but we suggest that the critical process of conversion to disease status in HD may be associated with relatively large, irreversible neuronal death predominantly in the caudate as suggested by MRI findings (Table 3, Figs 1B and 3). The HD process is a continuum in which neurodegenerative changes start long before the appearance of motor symptoms. Though discrete clinical stages of disease progression are defined by formal assessment systems such as TFC and disease stage, the biological disease process starts much earlier in gene-positive subjects, as demonstrated by fine MRI investigations, cognitive studies and functional MRI (fMRI) findings (Aylward et al., 2004; Reading et al., 2004; Rosas et al., 2005). With this in mind, the reduction of 24OHC appears to be associated with the disease severity in early HD and possibly marks the critical step of motor phenoconversion. In substance, we consider the pre-HD stage as a motor-silent stage of disease in which active neurodegeneration is in progress. In this context, 24OHC in plasma and MRI of the caudate appear to be suitable tools to follow this otherwise silent neurodegeneration in the pre-clinical stage, and 24OHC might be particularly appropriate to pinpoint phenoconversion. (Fig. 1A and B and Tables 2 and 3).

Albeit HD patients at any disease stage had significantly lower levels of plasma 24OHC than controls and pre-HD subjects, a fact arguably reflecting a reduced elimination of brain cholesterol in the form of 24OHC, we did not find evidence for reduction of 24OHC levels with disease progression from stage 1 to stage 3. We notice that pre-HD caudate volumes (absolute and TIV related) were significantly lower than controls. HD stages 1 and 2 had significantly reduced volumes compared with controls and pre-HD (Table S3 and Table 3). So, both MRI and 24OHC findings showed a similar pattern as to disease progression.

To our knowledge, this is the first evidence in a genetically defined disease, in which the process of neurodegeneration is well documented, for a brain-generated metabolite measurable in the plasma which is associated with definite neuronal losses. We do not have a unique molecular explanation for this finding, as it might be possible that: (i) it may reflect an accelerated disease-linked loss of metabolically active neurons associated with the process of phenoconversion from pre-manifest to early stage HD; (ii) it may represent a homeostatic reduction in brain 24OHC generation associated with decreased cholesterol synthesis, as previously shown in HD mice and human HD brains (Valenza et al., 2005, 2007b); (iii) it may mirror a combination of both mechanisms. In the absence of definitive data on cholesterol synthesis \textit{in vivo} in HD patients, we are inclined to favour the first explanation for the following reasons: (i) the long process leading to motor phenoconversion is associated with the loss of a critical mass of neurons in the striatum and the cortex; (ii) 24OHC levels in blood were directly correlated with a relative measure of neuronal loss as the decrease in caudate volume; (iii) in this perspective a correlative analysis of caudate volumes and 24OHC levels in controls, pre-manifest and HD1 and HD2 patients (total subject \( n = 72 \)), were consistent with 24OHC levels being directly correlated with neuronal cell loss \( (r = 0.41; P < 0.001, \text{Fig. S1}) \).

In conclusion our data demonstrate that: (i) 24OHC, a brain metabolite measurable in plasma, was shown to parallel a critical phase of neuronal loss in a genetic disease associated with progressive brain atrophy; (ii) 24OHC levels in blood appear to be a valuable tool to discriminate pre-manifest subjects from HD1 patients, and might therefore be helpful to investigate subjects close to the onset of motor manifestations; (iii) plasma 24OHC levels may complement MRI morphometry in monitoring neurodegenerative changes in pre-manifest and early Huntington disease. Now larger cross-sectional and longitudinal studies in pre-HD subjects and in HD patients are essential to validate further these data and their clinical implications.

**Supplementary material**

Supplementary material is available at \textit{Brain} online.

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References