Evidence for altered cholesterol metabolism in Huntington's disease post mortem brain tissue

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Abstract

Aims Cholesterol plays an essential role in membrane structure and function, being especially important in the brain. Alteration of brain cholesterol synthesis and metabolism has been demonstrated in several Huntington's disease (HD) mouse and cell models; however, less is known about these alterations in human tissue. This study aimed to identify alterations to cholesterol synthetic and metabolic pathways in human HD brain tissue. Methods A broad range of cholesterol synthetic precursors, metabolites and oxidation products were measured by gas chromatography-tandem mass spectrometry in five regions of human post mortem HD brain and compared with age- and sex-matched control tissues. The level of enzymes that regulate cholesterol homeostasis, cholesterol 24-hydroxylase and delta(24)-sterol reductase were investigated by Western blotting and qPCR in putamen. Results The most significant changes were localized to the putamen, where a 60% decrease in 24(S)-hydroxycholesterol, 30% increase in cholesterol and 100-200% increase in synthetic precursors (lathosterol, zymosterol and desmosterol) was detected. The enzymes cholesterol 24-hydroxylase and delta(24)-sterol reductase were also significantly decreased in HD putamen as compared with control tissues. Free radical-generated cholesterol oxidation products 7-keto cholesterol and 7β-hydroxycholesterol were also increased by 50-70% in HD putamen. Conclusion Human HD brain has significantly decreased cholesterol metabolism and disrupted cholesterol homeostasis. Our data also indicate that lipid oxidative stress accompanies HD pathology.

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Evidence for altered cholesterol metabolism in Huntington’s disease post-mortem brain tissue

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Running title: Altered cholesterol metabolism in Huntington’s disease

Abstract

**Aims:** Cholesterol plays an essential role in membrane structure and function, being especially important in the brain. Alteration of brain cholesterol synthesis and metabolism has been demonstrated in several Huntington’s disease (HD) mouse and cell models, however less is known about these alterations in human tissue. This study aimed to identify alterations to cholesterol synthetic and metabolic pathways in human HD brain tissue.

**Methods:** A broad range of cholesterol synthetic precursors, metabolites and oxidation products were measured by GC-MS/MS in five regions of human post-mortem HD brain and compared to age- and sex-matched control tissues. The level of enzymes that regulate cholesterol homeostasis, cholesterol 24-hydroxylase and delta(24)-sterol reductase were investigated by Western blotting and qPCR in putamen.

**Results:** The most significant changes were localised to the putamen, where a 60% decrease in 24(S)-hydroxycholesterol, 30% increase in cholesterol and 100 to 200% increase in synthetic precursors (lathosterol, zymosterol and desmosterol) was detected. The enzymes cholesterol 24-hydroxylase and delta(24)-sterol reductase were also significantly decreased in HD putamen as compared to control tissues. Free radical-generated cholesterol oxidation products 7-keto cholesterol and 7β-hydroxycholesterol were also increased by 50 to 70% in HD putamen.

**Conclusion:** Human HD brain has significantly decreased cholesterol metabolism and disrupted cholesterol homeostasis. Our data also indicates that lipid oxidative stress accompanies HD pathology.
Abbreviations:

HD = Huntington’ disease, GC-MS = gas chromatography-mass spectrometry, HTT = huntingtin, DHCR24 = delta(24)-sterol reductase, BBB = blood brain barrier, 24-OHC = 24(S)-hydroxycholesterol, CYP46A1 = cholesterol 24-hydroxylase, 27-OHC = 27-hydroxycholesterol, CYP27A1 = cholesterol 27-hydroxylase, CYP7B1 = 5-hydroxycholesterol 7-α-hydroxylase, AD= Alzheimer’s disease, PD = Parkinson’s disease, ROS = reactive oxygen species, COPs = cholesterol oxidation products, 7β-OH = 7β-hydroxycholesterol, 7-KC = 7-ketocholesterol, BDNF = brain derived neurotrophic factor.

Introduction

Huntington’s disease (HD) is an autosomal dominant, progressive neurodegenerative disease characterised by the expansion of a glutamine repeat on the N-terminus of the huntingtin protein (HTT). Classic symptoms include involuntary movement and cognitive dysfunction. The classical neuropathological hallmark of HD is the severe atrophy of the striatum (caudate and putamen) [1], with substantial volume loss in the order of 50%. While these brain regions are most severely affected, MRI techniques have highlighted that the hippocampus, cerebral cortex, globus pallidus and amygdala also have reduced volume in HD patients [2]. Loss of neurons is accompanied by progressive astrocytosis and an increased density of oligodendrocytes [3]. While it is not fully understood how the polyglutamine expansion causes cellular dysfunction in HD, it has been associated with lipids, altering membrane order [4] and the interaction with phospholipids [5]. Despite the genotypic identification of mutant huntingtin carriers, there is a lack of reliable biomarkers to predict HD progression or effectiveness of therapies. Several studies have identified that cholesterol synthesis and metabolism in HD cell lines and animal models is significantly disturbed [6-8], but the
mechanisms and metabolic pathways affected have not been fully examined in human HD brain.

Cholesterol is highly concentrated in the brain, accounting for 25% of total body cholesterol. The majority of cholesterol is found in myelin, accounting for 70% of total cholesterol, with the remainder found in cellular membranes of neurons and glial cells. Cholesterol has an essential role in many neurological processes including synaptogenesis [9], axon growth [10] and maintenance of dendrites [11]. Structurally, cholesterol modulates membrane fluidity and organisation [12] where it can alter signalling functions, membrane protein organisation and lipid raft structure [13-16]. Brain levels of cholesterol are tightly regulated by de novo synthesis, a pathway that involves over 20 steps. In the late stage of cholesterol synthesis the pathway branches at lanosterol into the Bloch pathway via the precursor desmosterol, or the Kandutsch-Russell pathway via lathosterol. Several other post squalene precursors exist in these pathways (see Fig. 1). Plasma levels of synthetic precursors have been positively correlated to whole body cholesterol synthesis [17, 18], but this does not reflect cholesterol synthesis in the central nervous system. Direct measurement of precursor levels in brain tissue is therefore necessary to monitor the cholesterol synthetic pathway [7, 8, 19]. Defects in the cholesterol synthetic pathway can have severe consequences, especially in the brain where tight regulation of cholesterol synthesis is essential for normal neuronal function. Mutations in downstream synthetic enzymes such as delta(24)-sterol reductase (DHCR24) can cause desmosterolosis [20], a severe developmental and neurological disorder.

The blood brain barrier (BBB) is impermeable to cholesterol, preventing fluctuations in circulating cholesterol affecting brain levels. The hydroxylation of cholesterol to more polar oxysterols enables movement across the BBB. 24(S)-Hydroxycholesterol (24-OHC), formed
by the brain specific enzyme cholesterol 24-hydroxylase (CYP46A1) has been identified as the major route of cholesterol elimination from the human brain [21]. Since CYP46A1 expression is primarily localised to neurons [22], it has been suggested that generation of 24-OHC (as measured in plasma) is a marker of metabolically active neurons in the brain [23]. Plasma 24-OHC levels are reduced in HD patients [24, 25] and recent evidence suggests that changes in CYP46A1 and 24-OHC brain levels may play a role in neurodegeneration [26-29]. However, the exact role this might play in HD pathogenesis has not been established.

Oxysterols formed in peripheral tissue may also enter the brain. C27 hydroxylation of cholesterol forms 27-hydroxycholesterol (27-OHC), an enzymatic reaction that occurs in many tissues outside the central nervous system. A concentration gradient results in a net movement of 27-OHC from circulation into the brain [30], where it is quickly metabolised into more polar products (including dihydroxysterols and cholestenoic acids), catalysed by the enzymes cholesterol 27-hydroxylase (CYP27A1) and 5-hydroxycholesterol 7-α-hydroxylase (CYP7B1) [31]. Accumulation of 27-OHC has been described in cases of Alzheimer’s disease (AD) [32] as well as patients with hereditary defects in the CYP7B1 gene [33], but very little is known regarding 27-OHC changes in HD.

Accumulation of unrepaired oxidative damage to biological macromolecules is a major component of neurodegenerative diseases, including AD [34] and Parkinson’s disease (PD) [35]. Oxidative stress is also observed in HD with markers of lipid peroxidation and DNA oxidation elevated [36-38]. Brain lipids represent a major target for oxidation, particularly cell membrane cholesterol that is susceptible to reactive oxygen species (ROS) attack and formation of cholesterol oxidation products (COPs). 7β-Hydroxycholesterol (7β-OHC) and 7-ketocholesterol (7-KC) are formed by direct ROS attack at the 5,6 double bond on cholesterol, and are elevated in diseases and pathological models that involve oxidative stress including atherosclerosis [39, 40], cystic fibrosis [41] and retinal photodamage [42]. Due to
the large pool of cholesterol in the brain, COPs represent potentially important biomarkers for neurodegenerative diseases.

Perturbed cholesterol pathways have been previously reported in HD mouse and cell models [6, 7, 19, 43]. While these models have described significant alteration of cholesterol synthesis and metabolism, the current literature contains very limited data obtained from human tissue, which is required for greater understanding of human HD pathophysiology. Here we report for the first time the level of cholesterol metabolites, synthetic precursors and oxidation products in 13 cases of human HD across 5 brain regions. We have also investigated the levels of two key cholesterol synthetic and metabolic enzymes in HD putamen.
**Materials and Methods**

**Materials**

Desmosterol-d$_6$, zymosterol-d$_5$, zymosterol and lanosterol-d$_6$ were obtained from Avanti lipids (Alabaster, AL, USA). Tert- butylhydroxytoluene (BHT), cholesterol, $\alpha$-cholestane, 7$\beta$-hydroxycholesterol, 7-dehydrocholesterol and 7-ketocholesterol and squalene were from Sigma (St. Louis, MO, USA). Lathosterol, lanosterol, desmosterol, 27-hydroxycholesterol, campesterol, and were obtained from Steraloids (Newport, RI, USA). Campesterol-d$_3$, 7$\beta$-hydroxycholesterol-d$_7$, lathosterol-d$_4$ and 7-ketocholesterol-d$_7$ were purchased from CDN Isotopes (Quebec, Canada). 27-hydroxycholesterol-d$_5$, 24-hydroxycholesterol and 24-hydroxycholesterol-d$_7$ were from Medical Isotopes , Inc (Pelham, AL, USA). Squalene-d$_6$ and 24,25-dihydrolanosterol-d$_6$ was obtained from Toronto research chemicals (TRC, Ontario, Canada). All standards obtained were of the highest purity (>95%). Methanol, hexane, methyl tert-butyl ether (MTBE), acetonitrile, toluene, formic acid and NaOH were purchased from Ajax Finechem (Thermo Fisher Scientific, AU). CUQAX223 UCT Clean-Up QAX2 solid phase extraction columns and BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) + 1% TMCS (trimethylchlorosilane) was purchased from PM Separations (Qld, Australia).

**Human brain tissue**

Frozen brain tissues from five brain regions (putamen, caudate, cerebellum, grey and white frontal cortex) were received from the Victorian Brain Bank Network. The cohort contained 13 cases of HD and 13 controls from each brain region. Tissue was transported on dry ice and stored at -80°C until analysis. Demographic and basic clinical data are presented in Table 1. The mean age of control cases was 68.9 ± 1.9 y which was not significantly different from
the HD cases with mean age of 67.3 ± 2.2 y. Post mortem interval (PMI) of control cases (41.5 ± 4 h) and tissue pH (6.4 ± 0.1) was not significantly different to HD PMI (37.5 ± 6.2 h) or tissue pH (6.4 ± 0.04). All brain tissue was from the left hemisphere of Caucasian donors. Ethics approval was from the University of Wollongong Human Research Ethics Committee (HE10/327). Control tissue was screened using standardized protocols to confirm the absence of degenerative pathologies. The research was carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association. All persons gave their informed consent prior to their inclusion in the study.

*Lipid extraction*

Brain lipids were extracted as previously described with minor modifications [44]. Frozen brain tissue (~5-10 mg) was weighed directly into a 0.5 mL polypropylene tube containing 5 Zircosil® ceramic beads (1.3 mm) (Klausen Pty Ltd, NSW, Australia), 150 µL methanol (0.01% BHT) and internal standards (4°C). Tissue was homogenised at 4°C using a Precellys 24 homogeniser (Bertin Technologies) (2 x 20 s at 5,000 rpm) and the homogenate was transferred to a clean glass vial. The tube and ceramic beads were washed with 100 µL methanol (4°C) and was added to the homogenate with 250 µL of NaOH (1 M). The sample was hydrolysed at room temperature for 16 h in the absence of light and then acidified with 330 µL of 1 M formic acid. The sample was made up to a final volume of 3 mL (8% methanol, pH 4.5) by the addition of 2.2 mL milliQ water. Solid-phase extraction (SPE) was carried out on a 200 mg mixed C8/anion exchange quaternary amine column (CUQAX223, UCT Inc.) that had been preconditioned with 2 mL methanol and then 2 mL 40 mM formic acid buffer (pH 4.5). The lipid extract was loaded and the column washed with 2 mL methanol in 40 mM formic acid (40:60). The SPE column was dried with N₂ gas flow for 5
min. Sterols and oxysterols were eluted with 2 mL hexane followed by 2 mL hexane/MTBE (50:50).

**GC-MS sterol analysis**

GC-MS sterol analysis was performed as previously described with minor modifications [35]. The sterol/oxysterol fraction was dried under N\textsubscript{2} at 37°C and derivatised by the addition of 20 µL acetonitrile and 20 µL BSTFA + 1% TMCS for one hour at 37°C. Samples were dried under N\textsubscript{2} and immediately reconstituted in 40 µL toluene for GC-MS/MS analysis. Selective reaction monitoring (SRM) analysis of sterols/oxysterols was carried out on an Agilent 7000B triple quadrupole mass selective detector interfaced with an Agilent 7890A GC system gas chromatograph. Quantification was performed by Agilent Masshunter Quantitative software (V B.05.00) by comparison of specific SRM transitions with their heavy isotopes and using relative response factor (RRF) calibration. Cholesterol was quantified using the internal standard α-cholestane in a separate injection and chromatographic run.

**Western Blotting**

Brain tissue (~20 mg) was added to 250 uL ice cold radio-immunoprecipitation assay (RIPA; 50 mM Tris, 150 nM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100) buffer containing 1% protease inhibitor (P8340, Sigma) and homogenised at 4°C using a Precellys 24 homogeniser (Bertin Technologies) (2 x 20 s, 6000 rpm). The homogenate was centrifuged at 14 000 x g and the soluble fraction taken for SDS-PAGE. Homogenates were incubated at 95°C for 10 min with loading dye containing β-mercaptoethanol. Equal amounts of protein (90 µg) were loaded onto a 12% acrylamide gel and electrophoresed for 1 h at
150V. Proteins were then transferred onto a 0.4 µm nitrocellulose membrane (BIO-RAD, Gladesville, NSW, Australia) for 35 min at 100V. Antigen retrieval was performed by adding 50 mL of boiling phosphate buffered saline + tween 20 (PBST) to the membrane and left to cool to room temperature. The membrane was blocked with a 10 mL solution of PBST containing 5% skim milk powder, rocking for 1 h at room temperature. Membranes were probed with antibodies detecting CYP46A1 1:100 (1F11, Santa Cruz Biotechnology) and DHCR24 1:1000 (ab137845, Abcam). Antibodies were diluted in 10 mL PBST 5% skim milk powder and incubated with membranes overnight, rocking at 4°C. Membranes were washed 3 x 10 min with PBST at room temperature before being incubated for 1 h at room temperature with a species specific IgG-HRP conjugated secondary antibody (1:3000). Membranes were washed 3 x 10 min with PBST before chemiluminescent detection of signal. Membranes were stripped, washed and re-probe for β-actin (1:10 000) following the protocol outlined above. Signal intensity was quantified using ImageJ software V1.46r (National Institutes of Health, USA), and normalised for β-actin.

qPCR

The quantitative PCR of human brain tissue was performed as previously described [45] with minor modifications. Briefly, human tissue (~30mg) was added to 10 volumes of TRIzol reagent (wt:vol) and homogenised in a Precellys 24 homogeniser at 2 x 20 s at 5500 rpm. The RNA concentration and purity was determined spectrophotometrically with a Nanodrop 1000 (Thermo Scientific). Following the manufacturer’s protocol, 2 µg of total RNA was used to synthesise cDNA using a Tetro cDNA synthesis kit (Oligo dT18) (Bioline, Sydney, Australia). Quantitative PCR was performed using a Roche Lightcycler 480 using SensiFAST SYBR No-ROX kit (Bioline) following the manufacturer’s instructions. Analyses were carried out in
triplicate and gene of interest mRNA was normalised to GAPDH and U6 mRNA levels. Gene expression was calculated using the comparative threshold cycle (Ct) value method using the formula $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct$ sample – $\Delta Ct$ reference) as described [46]. All primers were purchased from Sigma Aldrich (Sydney, Australia). The primer sequences used are as follows: $CYP46A1$ (F: TTCTAGGACACCTCCCCCTGC and R: CAGGTCCATACTTCTTAGCCCAAT) $DHCR24$ (F: TGTTCTGTGCTCTCCTCTCTC and R: ATTCCCCGACCTGCTTCTG) $GAPDH$ (F: GAGCACAAGAGGAAGAGAGAGGACCC and R: GTTGAGCACAGGGTACTTTATTGATGGTACATG) $U6$ (F: CTCGCTTCCGAGCAGCACA and R: AACGCTTCACGAATTTTCGT).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software V5.00 (Graphpad Software Inc., USA). An unpaired t-test was used to test for significantly different means, an F-test was used to determine if variances were significantly different. Welch’s correction for unequal variances was used when variances were found to be significantly different. Additional post hoc adjustments for multiple comparisons were conducted using the Benjamini and Hochberg method [47] in JMP software V12.1.0 (SAS Institute Inc., USA). All results are expressed as mean ± SEM with an adjusted $P < 0.05$ considered significant. A full list of $P$-values and adjusted $P$-values can be found in Supplemental Table 1.

Results

Human HD post mortem brain tissue from putamen, caudate, frontal cortex (grey and white) and cerebellum was analysed using mass dilution GC-MS/MS. Cholesterol metabolites,
oxidation products and synthetic precursors are presented as percent relative change compared to control (Fig. 2). Absolute values of sterols measured are provided in Supplemental Table 2.

**Cholesterol metabolites**

The most significant changes were detected in HD putamen, a region that is severely affected in HD. The brain specific cholesterol metabolite 24-OHC, was reduced close to 3-fold (p = 0.0011) in HD putamen (Fig. 2A). Caudate was the only other region to show changes in this metabolite with a 3-fold reduction (p = 0.0099) of 24-OHC in HD tissue compared to control tissue (Fig. 2A). 27-OHC, a peripheral cholesterol metabolite was significantly increased by 3-fold in HD putamen (p = 0.0196).

**Cholesterol oxidation products**

HD putamen was the only brain region to show evidence of oxidative stress, with a 50 to 70% increase in the cholesterol oxidation products 7-KC (p = 0.00495) and 7β-OH (p = 0.0355) (Fig. 2B).

**Cholesterol synthetic precursors**

Altered cholesterol synthesis was evident in HD putamen which exhibited increases in the cholesterol synthetic precursors, desmosterol (3-fold, p = 0.0140), lathosterol (2-fold, p = 0.0495), zymosterol (3-fold, p = 0.0145) and 24, 25 dihydro lanosterol (2-fold, p = 0.0140) (Fig 2C). Total cholesterol levels were also significantly increased by 30% in HD putamen (p
= 0.0470) (Fig. 2D). No other brain regions were found to have significant changes in cholesterol precursors, or cholesterol levels.

*Metabolic and synthetic enzymes*

To further investigate pathways that may contribute to changes in brain cholesterol metabolism and synthesis observed in putamen, we examined two major enzymes involved by western blotting. The protein level of CYP46A1 in HD was reduced 10-fold compared to control (p = 0.0027) (Fig. 3A). We also detected a significant (10-fold) reduction (p = 0.0014) of DHCR24 protein levels (Fig. 3B), which is in agreement with the accumulation of desmosterol seen in HD putamen (Fig. 2B).

The mRNA levels of the genes coding for cholesterol 24-hydroxylase (*CYP46A1*) and delta(24)-sterol reductase (*DHCR24*) were also measured in putamen using quantitative PCR. mRNA levels did not reflect protein levels and were not significantly changed between HD and control tissue in both *CYP46A1* and *DHCR2* (Fig. 4). mRNA levels normalised to individual house keeper genes can be found in Supplemental Figure 1.

**Discussion**

Alteration of cholesterol metabolism has been recently linked to several neurodegenerative diseases including AD, PD and HD [35, 43, 48]. In particular, the elimination pathway where 24-OHC is formed by the hydroxylation of cholesterol has been suggested as a contributing factor in neurodegeneration [49]. Neuronal 24-OHC has a major role in the turn-over of cholesterol in the brain [21], and the level of 24-OHC in plasma has been suggested as a measure of CYP46A1 enzymatic activity and thus metabolically active neurons [23].
Previous studies have found that the plasma levels of 24-OHC in HD patients correlate with disease severity and brain volume measured by MRI [24, 25]. Here for the first time we report the levels of 24-OHC in human HD post-mortem brain tissue. A significant reduction of 24-OHC was found in HD putamen along with a large (10-fold) reduction in the protein level of CYP46A1, the enzyme responsible for 24-OHC formation. Reduced enzyme levels may be due to neuronal loss, the location which CYP46A1 is primarily located. Alterations in post-transcriptional regulation may also play a role since no change in mRNA expression was detected between control and HD. This is potentially relevant as protein turnover is known to be dysregulated in HD cell models [50]. Caudate (another striatal region affected in HD) also had reduced levels of 24-OHC, while grey and white frontal cortex and cerebellum showed no change. Although the cerebral cortex is affected in later stages of HD [2, 51], the degree of volume loss and astrocytosis is substantially less compared to the striatum [2, 52, 53]. Reduced 24-OHC levels in HD striatum supports previous human studies that reported reduced plasma levels of this metabolite in HD patients [24, 25]. Our data indicates that decreased circulating 24-OHC levels are likely the result of reduced 24-OHC production in affected regions and not an aberration of BBB 24-OHC flux. In the mature brain, delivery of cholesterol synthesised in the astrocyte is required for normal neuronal function [9, 10, 54]. It has been hypothesised that 24-OHC production in neurons acts as a feedback molecule to initiate the delivery of cholesterol from astrocytes to neurons therefore maintaining homeostasis [55]. There is also evidence to suggest that CYP46A1 may have neuroprotective properties, being upregulated near plaques [27] and in glia of human AD brain [56]. Whether reduced levels of CYP46A1 in HD is a secondary event reflecting active neuron loss, or is a pathogenic factor altering cholesterol metabolism and synthesis remains to be established.

A defect in cholesterol synthesis has been previously described in several cell and mouse models of HD. Specifically; a reduction in the cholesterol synthetic precursors lathosterol and
lanosterol as well as cholesterol in the YAC128 mouse [19], reduced levels of lathosterol and lanosterol but not cholesterol in the R6/2 mouse model [7], reduced mRNA levels of cholesterol synthetic enzymes in R6/2 mouse and human HD fibroblasts [6] and reduced cholesterol synthetic enzymes in a transgenic HD cell model [43]. In contrast to these findings, our data in end stage human HD putamen describes an increase in cholesterol synthetic precursors from both the Bloch (desmosterol and 24,25 diHydro lanosterol) and Kandutsch-Russell (lathosterol and zymosterol) pathways and an increase in total cholesterol levels. No significant changes in squalene levels were observed (Suppl. Table 2) suggesting that the earlier mevalonate pathway did not influence the changes that we measured later in the pathway. The increase in cholesterol and synthetic precursors was only observed in the putamen; a region that degenerates early and severely in the human disease [57]. In contrast to macro and microscopic classification of disease pathology which sees the caudate degenerate consistently with the putamen [53, 57], we did not observe any cholesterol synthetic alterations in this region. While the cholesterol synthetic changes observed in the putamen may suggest an overall increase of cholesterol synthesis utilising both pathways, we believe this is not the case for the Bloch pathway. In our study, HD putamen had significantly higher levels of desmosterol, an immediate synthetic precursor of cholesterol and a substrate of DHCR24. Significantly depleted protein levels of DHCR24 in human HD putamen (10-fold) provides convincing evidence to explain the reduced DHCR24 activity and subsequent accumulation of desmosterol. Unaltered mRNA levels of DHCR24 in putamen again suggests enzyme levels are being affected post-transcription. Previous in vitro examination of cholesterol synthesis in specific cell types found astrocytes utilise the Bloch pathway via desmosterol, while neurons primarily utilised 7-dehydro cholesterol and other precursors from the Kandutsch-Russell pathway [58]. This suggests that in late stage human HD, desmosterol accumulates in astrocytes due to down regulated DHCR24. An increased density
of oligodendrocytes and activated astrocytes is known to be present in HD [3] and is believed to be a compensatory response to demyelination in HD [59]. While the specific cell type/s exhibiting elevated Kandutsch-Russell synthetic precursors is yet to be determined, increased oligodendrocytes attempting remyelination in affected tissue may contribute to this.

Previous studies in HD mouse brain reported a significant reduction of the cholesterol precursor lathosterol, which is found in the Kandutsch-Russell pathway. In contrast, the Bloch pathway precursor desmosterol, was either unchanged [19] or not reported [7] in these previous HD mouse studies. Examining precursors from both arms of the cholesterol synthetic pathway in these mouse models enables greater insight into the alterations occurring in different cell types, and their possible response to HD pathology / neurodegeneration. The differences seen between HD mouse models, in vitro cell models, and human post-mortem tissue may reflect a difference in the homeostatic response to mutant HTT between organisms and cell types. The differences in the data derived from our current study and previous mouse models and cell culture models may also reflect that in this study we examined end stage HD where a significant number of vulnerable neurons are predicted to have been lost. Examining earlier stages of the disease in brain tissue is therefore important to consolidate the role of cholesterol synthesis and metabolism in the early stages of HD in humans.

27-Hydroxycholesterol, a metabolite of cholesterol that is produced predominantly in peripheral tissue, was increased in the putamen of human HD brain as compared to controls. A trending increase was observed in grey cortex and cerebellum; however this lost significance after $P$-value adjustment. While an increased flux of 27-OHC into the brain may be caused by a disrupted BBB, we believe this is not the case in this tissue since sterols that
are present in higher peripheral abundance such as campesterol (a dietary derived phytosterol) were not significantly different between HD and control brain tissues (data not shown). As there is a net movement of 27-OHC from circulation into the brain [30], 27-OHC accumulation may be the result of a decrease in the activity of enzymes (such as CYP27A1) that further metabolises this oxysterol to cholestenoic acids [31]. Accumulation of 27-OHC is potentially important in neurodegeneration as *in vitro* studies have shown 27-OHC promotes β-amyloidogenesis [60] as well as being elevated in human AD brain and transgenic mice [32, 61]. While there is little direct *in vivo* physiological evidence that cholesterol metabolites promote neuronal damage, further studies are required to investigate the hypothesis that preventing the accumulation of 27-OHC in the brain reduces neurodegeneration in AD and HD.

Along with measuring cholesterol metabolites and precursors, our GC-MS method was able to sensitively detect oxidation products of cholesterol, and use these as markers of oxidative stress in HD brain tissue. Oxidative stress has been previously shown in HD [36], and in several other neurodegenerative diseases [34, 35, 62]. Similar to changes seen in other sterols, significant increases in 7-KC and 7β-OH were only observed in putamen. The specific increase in oxidation products in the putamen is consistent with this region being affected early and severely in HD [57]. 7-KC and 7β-OH have been shown to be stable, and previously used as markers of oxidative stress in plasma [40] and CSF [63]. Measurement of cholesterol oxidation in HD plasma together with other sterols may represent a convenient tool to follow progression, quantify severity, and assess therapeutic effectiveness in patients. Importantly, these markers can be measured sensitively and reliably by GC-MS techniques.
Homeostasis of cholesterol is essential for neurological function however the precise role of cholesterol in HD neurodegeneration is still debated. Evidence suggests that cholesterol accumulation in HD alters membrane organisation and cell signalling, enhancing the susceptibility of striatal neurons to excitotoxicity [4]. Another hypothesis suggests that impaired cholesterol synthesis drives neurodegeneration by limiting the supply of cholesterol to neurons [19]. As a result synaptogenesis and dendrite outgrowth is impaired [9, 11], and neurotransmission is disrupted [64]. It is also believed that a reduction of brain derived neurotrophic factor (BDNF) in HD [65] may play a significant role in neurodegeneration [66]. BDNF is a neurotrophin expressed by neurons which promotes cell survival, differentiation and growth [67]. Among these properties, BDNF has been shown to promote DHCR24 expression [68]. Therefore, a loss of BDNF action may also contribute to the cholesterol homeostatic imbalance we have detected in HD putamen.

Our study identifies for the first time that several cholesterol synthetic and metabolic pathways are disturbed in multiple brain regions of human HD, particularly putamen which plays a central role in HD pathophysiology. These data provide evidence to support previous studies that link altered cholesterol synthesis, metabolism and oxidative stress with the neuropathological process involved in HD. Since HD shares similar sterol disturbances as other late-onset neurodegenerative diseases, we believe these changes represent potential biomarkers for neurodegenerative disease and elucidating cholesterol related mechanisms of neuropathology may provide targets for therapeutic intervention. The key finding of reduced CYP46A1 in human HD brain confirms previous studies in HD patient plasma that indicate 24-OHC as a promising peripheral biomarker to monitor the development of HD neuropathology.
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Conflict of interest

The authors declare that they have no conflict of interest.

References


63. Leoni V, Lutjohann D, Masterman T. Levels of 7-oxocholesterol in cerebrospinal fluid are more than one thousand times lower than reported in multiple sclerosis. *J Lipid Res*. 2005; **46**: 191-5.


**Figure legends**

**Fig. 1** Simplified pathway showing cholesterol synthesis, metabolism and free radical oxidation relevant to this study. Major post squalene cholesterol synthetic precursors shown follow a branched pathway, the Kandutsch-Russell pathway or Bloch pathway. Cholesterol can be oxidised enzymatically to form 24(S)-hydroxycholesterol (24-OHC) or 27-hydroxycholesterol (27-OHC) by cholesterol 24-hydroxylase (CYP46A1) and cholesterol 27-hydroxylase (CYP27A1). Reactive oxygen species (ROS) can oxidise cholesterol to form 7-ketocholesterol and 7β-hydroxycholesterol. The position of delta(24)-sterol reductase (DHCR24), a cholesterol synthetic enzyme is also shown. Broken lines indicate intermediates that have not been shown in this simplified scheme.

**Fig. 2** Sterol levels in human HD putamen, caudate, grey cortex, white cortex and cerebellum. (A) Cholesterol metabolites (B) Cholesterol oxidation products (C) Cholesterol synthetic precursors (D) Cholesterol were measured by GC-MS/MS. Levels are expressed as a percentage change against control tissue. n = 12-13 per group, error bars represent +SEM. **p < 0.005, *p < 0.05.

**Fig. 3** Protein level of cholesterol synthetic and metabolic enzymes in human putamen. Brain tissue homogenates from HD and control were probed for (A) cholesterol 24-hydroxylase (CYP46A1) and (B) delta(24)-sterol reductase (DHCR24) by western blotting. Representative blots are shown (n = 3 per group). Integrated optical density for all blots (n = 9 per group) is represented as relative abundance compared to control (assigned a value of 1.0). Blots were normalised to β-actin level. Error bars represent +SEM. **p < 0.005.

**Fig. 4** mRNA levels of genes coding for cholesterol synthetic and metabolic enzymes in human putamen. The level of CYP46A1 (coding for cholesterol 24-hydroxylase) and DHCR24 (coding for delta(24)-sterol reductase) in brain tissue was determined by qPCR (n =
The genes of interest were normalised to GAPDH and U6 mRNA levels and expressed relative to control (assigned a value of 1.0). Error bars represent +SEM.
Figure 1
Figure 2
Figure 3

A

55kDa →

β-actin

Cholesterol 24-hydroxylase protein level

B

55kDa →

β-actin

24-Dehydrocholesterol reductase protein level
Figure 4

CYP46A1 mRNA levels

DHCR24 mRNA levels

Relative abundance

Control  HD  Control  HD