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Defective cholesterol metabolism in amyotrophic lateral sclerosis

Jonas Abdel-Khalik[1], Eylan Yutuc[1], Peter J. Crick[1], Jan-Åke Gustafsson[2], Margaret Warner[2], Gustavo Roman[3], Kevin Talbot[4], Elizabeth Gray[4], William J. Griffiths[1], Martin R. Turner[4], Yuqin Wang[1]

[1]Swansea University Medical School, Singleton Park, Swansea SA2 8PP, UK.

[2]Department of Biology and Biochemistry, Center for Nuclear Receptors and Cell Signaling, University of Houston, 3517 Cullen Blvd, Houston, TX 77204, USA.

[3]Methodist Neurological Institute, Methodist Hospital, Houston, TX 77030, USA.

[4]Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK.

Corresponding authors: Dr William J. Griffiths, Swansea University Medical School, Singleton Park, Swansea SA2 8PP, UK, Tel. +44 1792 295562, E-mail w.j.griffiths@swansea.ac.uk: Dr Martin R. Turner, Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK, Tel. +44 1865 223380, E-mail martin.turner@ndcn.ox.ac.uk: Dr Yuqin Wang, Swansea University Medical School, Singleton Park, Swansea SA2 8PP, UK, Tel. +44 1792 602730, E-mail y.wang@swansea.ac.uk.

Running title: Defective cholesterol metabolism in ALS

Abbreviations: ABC, ATP-binding cassette; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APO, apolipoprotein; BBB, blood brain barrier; CH25H, cholesterol 25-hydroxylase; CSF, cerebrospinal fluid; CYP, cytochrome P450; DHC, dehydrocholesterol; diHC, dihydroxycholesterol; EADSA, enzyme-assisted derivatisation for sterol analysis; FWHM, full-width at half maximum height; GC, gas chromatography; GP, Girard P; HC, hydroxycholesterol; HCO, hydroxycholestenone; HSD, hydroxysteroid dehydrogenase; LC, liquid chromatography; LXR, liver X receptor; MS, mass spectrometry; PLS, primary lateral sclerosis; SD, standard deviation; SPE, solid phase extraction; 25-D_3, 25-hydroxyvitamin D_3.

Abstract

As neurons die cholesterol is released in the central nervous system (CNS), hence this sterol and its metabolites may represent a biomarker of neurodegeneration, including in amyotrophic lateral sclerosis (ALS) in which altered cholesterol levels have been linked to prognosis. More than 40 different sterols were quantified in serum and cerebrospinal fluid (CSF) from ALS patients and healthy controls. In CSF the concentration of cholesterol was found to be elevated in ALS samples. When CSF metabolite levels were normalised to cholesterol, the cholesterol metabolite 3β,7α-dihydroxycholest-5-en-26-oic acid, along with its precursor 3β-hydroxycholest-5-en-26-oic acid and product 7α-hydroxy-3-oxocholest-4-en-26-oic acid were reduced in concentration, whereas metabolites known to be imported from the circulation into the CNS were not found to differ in concentration between groups. Analysis of serum revealed that (25R)26-hydroxycholesterol, the immediate precursor of 3β-hydroxycholest-5-en-26-oic acid was reduced in concentration in ALS patients compared to controls. We conclude that the acidic branch of bile acid biosynthesis, known to operative in-part in brain, is defective in ALS leading to a failure of the CNS to remove excess cholesterol which may be toxic to neuronal cells, compounded by a reduction in neuroprotective 3β,7α-dihydroxycholest-5-en-26-oic acid.

Keywords: Oxysterols; Mass spectrometry; Cytochrome P450; Nuclear receptors/LXR; Brain Lipids; Bile acids and salts/Biosynthesis; Cholestenoic acids; Neurodeneneration.

Introduction

Amyotrophic lateral sclerosis (ALS) is a heterogeneous, progressive and fatal neurodegenerative disease characterised by variable loss of upper and lower motor neurons (1). Biomarkers that are sensitive to the progression of disease have the potential to shorten therapeutic trials and provide new drug targets. Study of the metabolome offers the potential to identify disease-specific patterns for ALS, possibly providing such biomarkers and new insights into the deranged biochemical pathways associated with ALS. Metabolomic studies in blood and cerebrospinal fluid (CSF) have been performed utilising proton nuclear magnetic resonance spectrometry (2-4), gas chromatography – mass spectrometry (GC-MS) and liquid chromatography (LC) – MS (5, 6). Significant differences between patients and controls have been observed, consistent with a range of pathogenic mechanisms that have been described in ALS, including mitochondrial dysfunction, oxidative stress, excitotoxicity, neuroinflammation and hypermetabolism (7).

Lipids, distinct from other components of the metabolome (8), are non-polar or amphipathic in nature and require separate analysis from more water soluble metabolites. Cholesterol, both the non-esterified molecule and its esters with fatty acids, represent a major component of the total lipid content of cells in vertebrates. On a number of fronts, cholesterol and its metabolites, represent potential biomarkers for ALS. High plasma levels of cholesterol have been suggested to be neuroprotective for ALS and to be associated with an increased survival time (9-11), but other data suggests that accumulation of cholesterol esters and ceramides mediate oxidative-stress in motor neurons in ALS (12), while the gene cytochrome P450 27A1 (*CYP27A1*), encoding cholesterol (25R)26-hydroxylase (also known as sterol 27-hydroxylase), the first enzyme in the extra-hepatic part of the bile acid biosynthesis pathway, was recently identified as a susceptibility gene for sporadic ALS (13). In addition, statins, the cholesterol lowering drugs which inhibit of HMG-CoA reductase, have been suggested to accelerate functional decline in ALS patients (14).

ALS is a neurodegenerative disease and as neurons die cholesterol is released from cells. Cholesterol and its metabolites in the CNS are predominantly in a non-esterified form (15) and it the non-

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esterified molecules that are responsible for regulating cholesterol homeostasis (16, 17), are the ligands to nuclear receptors (18, 19) and ligands to G-protein coupled receptors (20, 21). We aimed to characterise and quantify non-esterified cholesterol and the widest range of non-esterified metabolites in CSF and serum from ALS patients compared to a group of healthy controls (see Table 1 for molecules analysed). A LC-MS approach was adopted exploiting charge-tagging to ensure maximum sensitivity and to gain structural information allowing identification of unexpected sterols (supplemental Fig. S1), a hydrolysis step was not included in the sample preparation protocol (22, 23).

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Materials and Methods

Human Samples

Serum was from 35 patients diagnosed with ALS (24 male, 11 female, mean age 65), 6 patients diagnosed with primary lateral sclerosis (PLS, 2 male, 4 female, mean age 69) and 24 control samples (12 male, 12 female, mean age 58). CSF was from ALS patients (n = 20, 15 male, 5 female, mean age 61) and controls (n = 15, 12 male, 3 female, mean age 75). Serum and CSF samples were obtained from ALS and PLS patients and healthy volunteers (typically patient spouses and friends) as part of The Oxford Study for Biomarkers in Motor Neuron Disease (BioMOx). Two expert neurologists made the diagnosis of ALS and PLS according to standard criteria (MRT, KT). All participants provided informed consent and the study was approved by the South Central Oxford Ethics Committee B. Additional control CSF samples for method development were from a study performed at Methodist Hospital, Houston.

Methods

Serum

100 µL of serum was added drop-wise to a solution of 1050 µL absolute ethanol containing 2 ng of 25-[26,26,26,27,27,27-^{2}H$_6$]hydroxyvitamin D$_3$ (25-[^{2}H$_6$]D$_3$, Toronto Research Chemicals, Ontario, Canada), 2 ng of 7α,25-[26,26,26,27,27,27-^{2}H$_6$]dihydroxycholesterol (7α,25-[^{2}H$_6$]diHC), 20 ng of 7α-[25,26,26,26,27,27,27-^{2}H$_7$]hydroxycholesterol (7α-[^{2}H$_7$]HC), 20 ng of 24R/S-[25,26,26,26,27,27,27-^{2}H$_7$]hydroxycholesterol (24R/S-[^{2}H$_7$]HC), 20 ng of 22R-[25,26,26,26,27,27,27-^{2}H$_7$]hydroxycholset-4-en-3-one (22R-[^{2}H$_7$]HCO) and 20 µg [25,26,26,26,27,27,27-^{2}H$_7$]cholesterol (deuterated sterols from Avanti Polar Lipids Inc, Alabaster, AL) with sonication in an ultrasonic bath. After 5 min, 350 µL of water was added to make the solution 70% ethanol. After a further 5 min of sonication the solution was centrifuged at 17,000 × g at 4°C for 30 min.

To separate cholesterol from the more polar oxysterols and steroid acids the sample solution, now in 1.5 mL of 70 % ethanol, was loaded onto a 200 mg Certified Sep-Pak C$_{18}$ column (Waters, Elstree,

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Herts, UK) pre-conditioned with 4 mL of absolute ethanol and with 6 mL of 70% ethanol. The solvent flow-rate through the column was at a rate of ~0.25 mL/min assisted by negative pressure at the column outlet generated by a vacuum manifold. The flow-through (1.5 mL) was combined with a column wash of 70% ethanol (5.5 mL) to give fraction SPE1-Fr1 (7 mL) in which 25-D_3, oxysterols, cholestenoic and cholenoic acids elute. A second fraction (SPE1-Fr2) was collected by eluting with a further 4 mL of 70% ethanol, before fraction 3 containing cholesterol and sterols of similar hydrophobicity was eluted with 2 mL of absolute ethanol (SPE1-Fr3). Finally, a fourth fraction eluting in 2 mL of absolute ethanol was collected containing lipid more hydrophobic than cholesterol (SPE1-Fr4). Each fraction was divided into two equal sub-fractions (A) and (B) and allowed to dry overnight under vacuum. Note, a hydrolysis step was not included in the sample preparation procedure and by removing cholesterol from its metabolites in the first step of sample preparation subsequent confusion with ex vivo oxidation products is eliminated.

Lyophilised material was reconstituted in 100 µL of propanol-2-ol. Each sample was vortexed thoroughly. To sub-fractions (A) 1000 µL of 50 mM phosphate buffer pH 7 was added containing 3.0 µL of cholesterol oxidase from Streptomyces sp (2 mg/mL in H_2O, 44 units/mg of protein, Sigma-Aldrich Ltd, Dorset, England). The reaction mixture was left at 37°C for 1 hour after which the reaction was quenched with 2000 µL of methanol. Sub-fractions (B) were treated identically to sub-fractions (A), but in the absence of cholesterol oxidase. 150 µL of glacial acetic acid was added to sub-fractions (A) and (B) and vortexed. 190 mg of [$^2H_5$]Girard P (GP) reagent, bromide salt (23), was added to sub-fractions (A), 150 mg of [$^2H_0$]GP reagent, chloride salt (TCI Europe, Zwijndrecht, Belgium), was added to sub-fractions (B) to allow for subsequent simultaneous LC-MS analysis, see below (supplemental Fig. S1). Following vortexing, the derivatisation reaction was left to proceed at room temperature, overnight in the dark.

To remove excess derivatisation reagent a 60 mg Oasis HLB (Waters) column was conditioned with 6 mL of 100 % methanol, 6 mL of 10 % methanol and 4 mL of 70 % methanol before the samples (3250 µL, ~70% organic) were loaded. The sample vessel was washed with 1 mL of 70 % methanol and the wash was applied to the column. 1 mL of 35 % methanol was used to rinse the column and the

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combined 5 mL of effluent was diluted with 4 mL of water to give 9 mL of 35 % methanol solution. This solution was re-applied to the column followed by a rinse of the column with 1 mL of 17.5 % methanol. The 10 mL of combined effluent was diluted with 9 mL of water to give a 19 mL solution of 17.5 % methanol. Re-applying the 19 mL solution to the column completed the recycling procedure by which sterols, oxysterols, cholestenoic and cholenoic acids were extracted by the column while derivatisation reagents eluted to waste. 6 mL of 10 % methanol was added to wash the column. Finally, 25-D$_3$, oxysterols, cholestenoic and cholenoic acids and more non-polar sterols were eluted from the column with 3 x 1 mL 100 % methanol (SPE2-Fr1,2,3) and 1 mL absolute ethanol (SPE2-Fr4). Oxysterols, 25-D$_3$, cholestenoic and cholenoic acids elute in the first 2 mL from the column (SPE2-Fr1,2). Cholesterol and sterols of similar polarity elute over 3 mL of methanol (SPE2-Fr1,2,3).

For 25-D$_3$, oxysterol, cholestenoic and cholenoic acid analysis, just prior to analysis by LC-MS an equal aliquot of SPE2-Fr1,2 from sub-fractions (A) and (B) were combined together in one tube. For cholesterol analysis equal volumes of SPE2-Fr1,2,3 from sub-fractions (A) and (B) were combined. In both cases the combined effluent in methanol was diluted with water to achieve a concentration of 60 % methanol, i.e. the initial composition of the mobile phases during LC analyses.

LC-MS and -MS^n analysis

Analysis was performed on a LTQ-Orbitrap Elite (Thermo Fisher Scientific, Hemel Hempstead, UK) equipped with an electrospray probe, and a Dionex Ultimate 3000 LC system (Dionex now Thermo Fisher Scientific), essentially as described previously (22, 23). For each injection three to five scan events were performed: one high resolution (120,000, FWHM at *m/z* 400) MS scan event in the Orbitrap analyser in parallel with two to four multi-stage fragmentation (MS^n) scan events in the LTQ linear ion trap. Quantification was performed by stable isotope dilution or using isotope labelled structural analogues.

CSF

250 µL of CSF was added drop-wise to 2100 µL of absolute ethanol containing 2 ng of 24R/S-[^{2}H$_7$]HC, 2 ng of 7α-[^{2}H$_7$]HC, 2 ng of 22R-[^{2}H$_7$]HCO, 0.4 ng of 7α,25-[^{2}H$_6$]diHC, 4 ng of 25-[^{2}H$_6$]D$_3$

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and 0.8 µg of [^{2}H$_7$]cholesterol with sonication in an ultrasonic bath. 650 µL of water was added and the solution sonicated for 5 min followed by centrifugation at 2,400 g at 4°C for 30 min.

The CSF sample solution now in 3 mL of 70% ethanol was loaded on to a 200 mg Certified Sep-Pak C$_{18}$ column as in the serum protocol. However, the 3 mL flow-through was combined with a column wash of only 4 mL of 70% ethanol to give SPE1-Fr1 (7 mL) in which 25-D$_3$, oxysterols, cholestenoic and cholenoic acids elute. The remainder of the sample preparation procedure including derivatisation was identical to that used for serum, with the exception that the final eluates from SPE2 were lypholised and reconstituted in 60% methanol prior to sample injection on the LC system.

Statistics

An ANOVA test was run for each sterol. Uni-variant t-tests were performed against the control group, *P < 0.05; **P < 0.01. Concentrations given in the text are mean ± standard deviation (SD).

Results

Analysis of sterols, oxysterols, cholestenoic and cholenoic acids in serum

Using enzyme-assisted derivatisation for sterol analysis (EADSA) technology (22) in combination with LC-MS with MS[3] we analysed serum from 35 patients diagnosed with ALS (24 male, 11 female, mean age 65) and 24 control samples (12 male, 12 female, mean age 58). Six patients diagnosed with the upper motor neuron-only, very slowly-progressive variant of ALS, termed PLS (2 male, 4 female, mean age 69) were separately compared to the control group. Concentrations of non-esterified cholesterol, two of its precursors and >40 metabolites were measured (see Table 1 for compounds and abbreviations and supplemental Table S1 for quantitative data, note a hydrolysis step was not carried out so all concentrations are for non-esterified compounds). We failed to find any sexual dimorphism in the control sample set and thus included both sexes in the current study.

There was no significant difference in the concentrations of cholesterol or its precursors desmosterol (24-DHC) or 7-dehydrocholesterol (7-DHC) between ALS, PLS or controls. Note we report here the concentration of 7-DHC as the sum of 7-DHC and its isomer 8-DHC which are only partially resolved on our LC system. 8-DHC is an enzymatic product of 7-DHC (24). Similarly, there was no significant difference in the concentration of the 7-DHC metabolite 25-hydroxyvitamin D_3 (25-D_3) in serum of the ALS patients compared to controls (Fig. 1).

The first steps of all cholesterol metabolism lead to hydroxycholesterol (HC) isomers, collectively known as oxysterols. A minor pathway, initiated in activated macrophages, leads to 25-hydroxycholesterol (25-HC) followed by subsequent metabolism to 7α,25-dihydroxycholesterol (7α,25-diHC) and 7α,25-dihydroxycholest-4-en-3-one (7α,25-diHCO, Fig. 2). Of these metabolites 7α,25-diHCO was elevated in ALS serum (P < 0.01). 7α-Hydroxycholesterol (7α-HC) and 7α-hydroxycholest-4-en-3-one (7α-HCO) are the first members of the neutral pathway of bile acid biosynthesis (Fig. 3) (25), however, neither oxysterol showed a difference in concentration between ALS, PLS or control samples. Neither did 7β-hydroxycholesterol (7β-HC) or 7-oxocholesterol (7O-

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C). 7O-C, 7α-HC and 7β-HC can be formed via free radical oxidation of cholesterol and these metabolites have been suggested as markers of oxidant stress (26).

On-the-other hand, (25R)26-hydroxycholesterol (26-HC), the first member of the extra-hepatic part of the acidic pathway of bile acid biosynthesis, was decreased in ALS and PLS serum compared to controls ($P < 0.01$), as was 3β-hydroxycholest-5-en-26-oic acid (3β-HCA) in ALS serum ($P < 0.05$). CYP27A1 is the enzyme responsible for hydroxylation of cholesterol at (25R)C-26 and then subsequent oxidation to the (25R)26 carboxylic acid. Note, we use the systematic nomenclature (27), where hydroxylation of the terminal carbon of the cholesterol side-chain introducing 25R stereochemistry results in (25R)26-hydroxycholesterol (28). Unless stated otherwise R stereochemistry is generally assumed at C-25. The non-systematic name for 26-HC, widely used in the literature on account of the nomenclature of its synthesising enzyme, CYP27A1, is 27-hydroxycholesterol (27-HC). Levels of 26-HC in the circulation are often correlated to cholesterol, with high cholesterol levels associated with high levels of 26-HC (29). We thus decided to normalise for each sample the level of 26-HC and other metabolites to cholesterol to find if the observed differences were still maintained. When normalised to cholesterol (supplemental Table S2), elevation in concentration of 7α,25-diHCO was retained in ALS, as was a decreases in the concentration of 26-HC. Interestingly, in an earlier study, Wuolikainen et al found that "total" 26-HC in male ALS patients was similarly reduced (30). In combination these results suggest reduced transcription, translation or activity of cholesterol (25R)26-hydroxylase in ALS.

Analysis of sterols, oxysterols, cholestenoic and cholenoic acids in CSF

As with serum, levels of cholesterol, 7-DHC (plus 8-DHC) and desmosterol were measured in CSF from ALS patients (n = 20, 15 male, 5 female, mean age 61) and controls (n = 15, 12 male, 3 female, mean age 75). The concentrations of both desmosterol ($P < 0.05$) and cholesterol ($P < 0.01$) were found to be elevated in CSF from ALS patients (Fig. 4, supplemental Table S3). As ALS is a neurodegenerative disease we speculated that when cholesterol is released by neurons as they die it would be metabolised by CYP46A1 to 24S-hydroxycholesterol (24S-HC) and by CYP27A1 to

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members of the acidic pathway of bile acid biosynthesis (Fig. 2 & 3, respectively) (25). Surprisingly, when normalised to cholesterol 24S-HC concentration was found to be reduced (P < 0.01) in CSF as were members of the acidic pathway of bile acid biosynthesis (supplemental Table S4). Intriguingly, concentrations of 7α-HC, 7α-HCO, 7β-HC and 7O-C which originate from the CYP7A1 initiated arm of the bile acid biosynthesis pathway and enter the CSF from the circulation did not differ between ALS patients and controls (Fig. 5). This can be explained as CYP7A1 is not expressed in the CNS, being liver-specific (Fig. 3) (25). The level of 26-HC was not significantly lower in CSF from ALS patients (0.09±0.06 ng/µg cholesterol) compared to controls (0.13±0.07 ng/µg cholesterol), but its downstream metabolites 3β-HCA (P < 0.01), 3β,7α-dihydroxycholest-5-en-26-oic acid (3β,7α-diHCA, P < 0.05) and 7α-hydroxy-3-oxocholest-4-en-26-oic acid (7αH,3O-CA, P < 0.01) were all reduced in concentration (ng/µg cholesterol) in ALS CSF (Fig. 6). While (25R)26-carboxylation by CYP27A1 occurs in the mitochondria, 7α-hydroxylation by CYP7B1, the oxysterol 7α-hydroxylase, and oxidation of the 3β-hydroxy group by hydroxysteroid dehydrogenase (HSD) 3B7 occur in the endoplasmic reticulum, further metabolism to bile acids proceeds in the peroxisome (31). Peroxisomal metabolites found in CSF include 7α,24-dihydroxy-3-oxocholest-4-en-26-oic acid (7α,24-diH,3O-CA), 7α-hydroxy-3,24-*bis*oxocholest-4-en-26-oic acid (7αH,3,24-diO-CA), which is also observed as the decarboxylated dione, 7α-hydroxy-26-*nor*cholest-4-ene-3,24-dione (7αH,26-nor-C-3,24-diO), and the beta-oxidation product 7α-hydroxy-3-oxochol-4-en-24-oic acid (7αH,3O-Δ^4-BA). With the exception of the latter compound all of the perxisomal intermediates were decreased (P < 0.05 or 0.01, ng/µL cholesterol) in CSF from ALS patients.

Discussion

The major route for cholesterol metabolism in the CNS is conversion to 24S-HC via the neuron specific enzyme CYP46A1 (32, 33), accounting for about two thirds of cholesterol metabolism in brain, and elevated levels of "total" 24S-HC have been found in CSF of patients with some neurodegenerative diseases e.g. Alzheimer's disease (AD) (34, 35). The term "total" describes the sum of both esterified and non-esterified sterol. In most studies concentrations of "total" cholesterol or of "total" hydroxycholesterols are measured where sterols esterified with fatty acids are hydrolysed and the "total" sterol assayed, although it is the non-esterified sterols which are biologically active. Usually the ratio of esterified to non-esterified sterol in plasma is about 10:1 (36). Surprisingly, levels of "total" 24S-HC are decreased in blood plasma of patients with advanced AD, presumably as a consequence of a reduced number of active neurons producing 24S-HC (37). In a recent study Wuolikainen et al found "total" 24S-HC plasma levels to be increased in female ALS patients but not in male patients, while "total" 26-HC was reduced in male ALS patients but not females (30). Side-chain hydroxycholesterols, i.e. 22R-hydroxycholesterol (22R-HC), 24S-HC, 25-HC, and 26-HC, and also cholestenoic acids, are ligands to liver X receptors (LXRs), the β isoform being particularly expressed in brain, and interestingly LXRβ-/- mice show adult-onset motor neuron pathology (38, 39). Importantly, LXRβ is involved in control of lipogenesis and cholesterol homeostasis and these mice showed increased cholesterol levels in spinal cord, gliosis, and inflammation preceding motor neuron loss and clinical disease onset (40). In the present study a loss of LXR signalling may result from reduced CNS levels of oxysterol and cholestenoic acid LXR-ligands, ultimately leading to motor neuron pathology.

While cholesterol cannot cross the blood brain barrier (BBB) and be imported to, or exported from, brain, 24S-HC, 26-HC and other cholesterol metabolites can cross the BBB (35, 41). Heverin et al have shown that 26-HC is imported to brain (42), while Meaney et al have shown that its acidic metabolite 7αH,3O-CA is exported from brain to the circulation (43). Further studies by Iuliano et al have shown that a precursor of this acid, 7α,26-dihydroxycholest-4-en-3-one (7α,26-diHCO) is also exported from brain to the circulation as is its isomer 7α,25-diHCO (44). Other studies by Ogundare

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et al have shown that multiple parts of the acidic pathway of bile acid biosynthesis are active in CNS (45).

A key finding of the present study was the increase in concentration of non-esterified cholesterol in the CSF of ALS patients (Fig. 4). This increase was associated with a decrease in the cholesterol-normalised concentration of brain-derived oxysterols and cholestenoic acids but not of those cholesterol metabolites imported from the circulation (Fig. 5 & 6). One explanation for increased cholesterol levels in CSF of ALS patients is that cholesterol is released from membranes as neurons die and metabolic pathways are saturated and insufficient to remove this excess in ALS patients (Figure 7). An alternative explanation is that there is dysregulated transport of sterols between neurons and glia resulting in inefficient cholesterol metabolism in the CNS of ALS patients as a consequence of ineffective LXR-signalling. This latter hypothesis is supported by studies on the LXRβ-/- mouse which not only shows adult motor neuron pathology but also increased spinal cord cholesterol levels, accumulation of cholesterol in ventral horn neurons, gliosis and inflammation preceding motor neuron loss and clinical disease onset (40). How LXRβ protects against motor neuron loss is not exactly clear, but LXRβ is known to attenuate the inflammatory response (46), and neuroinflammation is an important pathogenic factor in ALS (47). In fact, proinflammatory cytokines and monocyte chemotactic protein 1 were increased in LXRβ-/- mice, suggesting LXRβ plays a fundamental role in the CNS by reducing levels of cytokines and therefore development of neuroinflammation (40).

Importantly, the oxysterol 24S-HC and the cholestenoic acids 3β-HCA, 3β,7α-diHCA and 3β,7β-diHCA, each found to be reduced in CSF from ALS patients, are LXR ligands (48). Thus, neuroinflammation in ALS may be associated with a reduction in CNS LXR ligands and consequently LXR signalling. In fact, 3β,7α-diHCA has been ascribed a neuroprotective role towards developing motor neurons through the LXRs, although the other two acids appear to be neurotoxic (48). One explanation for reduced levels of LXR ligands in CSF of ALS patients is that as neurons die in ALS, less of the neuron-specific endoplasmic reticulum enzyme CYP46A1 is available to metabolise cholesterol to 24S-HC, thereby accounting for its reduced concentration in CSF. Other LXR ligands

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found in CSF, including 3β-HCA, 3β,7α-diHCA and 3β,7β-diHCA, are derived from cholesterol in astrocytes (49). The first enzyme in their synthesis from cholesterol is mitochondrial CYP27A1 (Fig. 3). Interestingly *CYP27A1* has been identified as a susceptibility gene for sporadic ALS (13). Mutations in *CYP27A1* leading to a defective CYP27A1 enzyme are the cause of the genetic neurodegenerative disorder cerebrotendinous xanthomatosis (50).

Further support for a role for oxysterols in neuroinflammation comes from the recent report by Reboldi et al linking side-chain hydroxylated cholesterol molecules (i.e. 25-HC) to inhibition of *Il1b* transcription and inflammasome activity by antagonising the SREBP-driven cholesterol biosynthesis pathway (51). Importantly, it has been shown that 24S-HC will also inhibit SREBP-driven cholesterol biosynthesis (52), so conversely reduced levels of 24S-HC in CNS, as observed in ALS, should result in enhanced cholesterol biosynthesis and *Il1b* transcription, inflammasome activity and neuroinflammation.

In summary, we have identified elevated cholesterol concentrations in the CSF of ALS patients. When normalised to cholesterol, concentrations of glial derived acidic metabolites are reduced, but cholesterol metabolites imported from the circulation are not. This data points to impaired activity of the CYP27A1 enzyme leading to a failure of the CNS to remove excess cholesterol which may be toxic to neuronal cells, compounded by a reduction in neuroprotective 3β,7α-diHCA and other LXR ligands (Fig. 7).

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Table 1. Sterols, oxysterols, cholestenoic and cholenoic acids analysed in serum and CSF

Systematic Name (trivial name)	Abbreviation	Lipid Maps ID
3β-Hydroxycholestatriene		NA
Cholesta-5,7-dien-3β-ol (7-Dehydrocholesterol) + Cholesta-5,8-dien-3β-ol	7-DHC + 8-DHC	LMST0101006 /NA
Cholesta-5,24-dien-3β-ol (Desmosterol)	24-DHC	LMST0105001
Cholest-5-en-3β-ol (Cholesterol)	C	LMST0105000
Cholesta-5,8-diene-3β,24(or 25)-diol	24(or 25)H-8DHC	NA
Cholesta-5,8-diene-3β,26-diol	26H-8-DHC	NA
3β-Hydroxycholest-5-en-7,8-epoxide	7,8-EC	NA
9,10-Secocholesta-5Z,7E,10-triene-3S,25-diol (25-Hydroxyvitamin D₃)	25-D₃	LMST03022024
9,10-Secocholesta-5Z,7E,10-triene-3S,24,25-triol (24,25-Dihydroxyvitamin D₃)	24,25-D₃	LMST03022027 LMST03022027
Cholest-4(or 5)-ene-3β,6β-diol (6β-Hydroxycholesterol)	6β-HC	NA
Cholest-5-ene-3β,7α-diol (7α-Hydroxycholesterol)	7α-HC	LMST0101001
7α-Hydroxycholest-4-en-3-one	7α-HCO	LMST0101027
Cholest-5-ene-3β,7β-diol (7β-Hydroxycholesterol)	7β-HC	LMST0101004
3β-Hydroxycholest-5-en-7-one (7-Oxocholesterol)	7O-C	LMST0101004
3β-Hydroxycholest-5-en-24-one (24-Oxocholesterol)	24O-C	LMST0101013
Cholest-5-ene-3β,24S-diol (24S-Hydroxycholesterol)	24S-HC	LMST0101001
Cholest-5-ene-3β,25-diol (25-Hydroxycholesterol)	25-HC	LMST0101001
Cholest-5-ene-3β,(25R)26-diol (27-Hydroxycholesterol)	26-HC	LMST0101005
Cholest-5-ene-3β,12α-diol (12α-Hydroxycholesterol)	12α-HC	NA
Cholest-5-ene-3β,7α,12α-triol (7α,12α-Dihydroxycholesterol)	7α,12α-diHC	LMST0403016
7α,12α-Dihydroxycholest-4-en-3-one	7α,12α-diHCO	LMST0403011
Cholest-5-ene-3β,7α,25-triol (7α,25-Dihydroxycholesterol)	7α,25-diHC	LMST0403016
7α,25-Dihydroxycholest-4-en-3-one	7α,25-diHCO	LMST0403010

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Cholest-5-ene-3β,7α,26-triol (7α,26-Dihydroxycholesterol)	7α,26-diHC	LMST0403008
7α,26-Dihydroxycholest-4-en-3-one	7α,26-diHCO	LMST0403015
3β,22-Dihydroxycholest-5-en-24-one	22-HC-24-O	NA
3β,5α-Dihydroxycholest-7-en-6-one (Dihydrocholestenone)		LMST0101026
Cholesta-5-en-3β,24,25-triol (24,25-Dihydroxycholesterol)	24,25-diHC	NA
3β-Hydroxycholest-5-en-26-oic acid	3β-HCA	LMST0403007
3-Oxocholest-4-en-26-oic acid	3O-CA	NA
3β-Hydroxycholesta-5,7-dien-26-oic acid	3βH-7-DHCA	NA
3-Oxocholesta-4,6-dien-26-oic acid	3O-6-DHCA	NA
3β,7α-Dihydroxycholest-5-en-26-oic acid	3β,7α-diHCA	LMST0403014
7α-Hydroxy-3-oxocholest-4-en-26-oic acid	7αH,3O-CA	LMST0403014
Total 3β,7α-Dihydroxycholest-5-en-26-oic acid	3β,7α-diHCA	LMST0403014
Total 7α-Hydroxy-3-oxocholest-4-en-26-oic acid	7αH,3O-CA	LMST0403014
3β,7β-Dihydroxycholest-5-en-26-oic acid	3β,7β-diHCA	NA
3β-Hydroxy-7-oxocholest-5-en-26-oic acid	3βH,7O-CA	LMST0403021
3β,22,25-Trihydroxycholest-5-en-24-one	22,25-diHC-24-O	NA
7α,x,y-Trihydroxycholest-4-en-3-one	7α,x,y-triHCO	NA
7α,24(or26),25-Trihydroxycholest-4-en-3-one	7α,24(or 26),25-triHCO	NA
3β,7α,24-Trihydroxycholest-5-en-26-oic acid	3β,7α,24-triHCA	NA
7α,24-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,24-diH,3O-CA	NA
3β,7β,24-Trihydroxycholest-5-en-26-oic acid	3β,7β,24-triHCA	NA
7β,24-Dihydroxy-3-oxocholest-4-en-26-oic acid	7β,24-diH,3O-CA	NA
3β,7α,25-Trihydroxycholest-5-en-26-oic acid	3β,7α,25-triHCA	NA
7α,25-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,25-diH,3O-CA	NA
3β,7β,25-Trihydroxycholest-5-en-26-oic acid	3β,7β,25-triHCA	NA
7β,25-Dihydroxy-3-oxocholest-4-en-26-oic acid	7β,25-diH,3O-CA	NA
7α,x-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,x-diH,3O-CA	NA
3β,7α,12α-Trihydroxycholest-5-en-26-oic acid	3β,7α,12α-triHCA	NA

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7α,12α-Dihydroxy-3-oxocholest-4-en-26-oic acid	7α,12α-diH,3O-CA	LMST0403015
Trihydroxy-3-oxocholest-4-en-26-oic acid		NA
7α-Hydroxy-3,24-bisoxocholest-4-en-26-oic acid	7αH,3,24-diO-CA	NA
7α-Hydroxy-26-nor-cholest-4-ene-3,24-dione	7αH,26-nor-C-3,24-diO	NA
3β,7α-Dihydroxychol-5-en-24-oic acid	3β,7α-Δ^5-BA	LMST0401021
7α-Hydroxy-3-oxochol-4-en-24-oic acid	7αH,3O-Δ^4-BA	LMST0401023
3β,7β-Dihydroxychol-5-en-24-oic acid	3β,7β-Δ^5-BA	LMST0401021

Notes

1. Location of double bonds unknown.

2. 7-DHC isomerises to 8-DHC.

3. Dehydration product of cholestane-3β,5α,6β-triol.

4. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

5. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

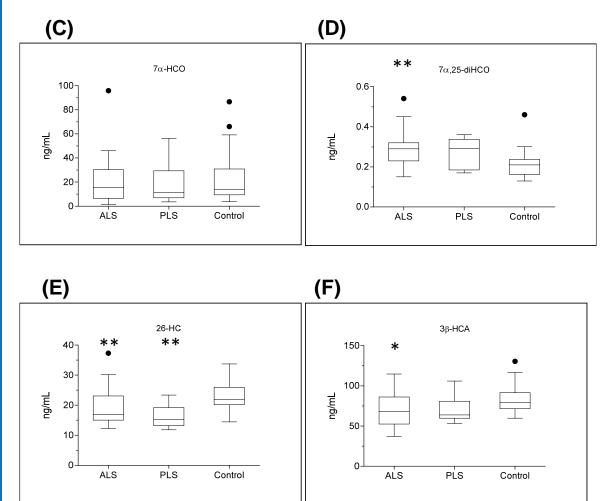6. 3β,7α-diHCA dehydrates to 3βH-7-DHCA.

7. 7αH,3O-CA dehydrates to 3O-6-DHCA.

8. Sum of intact 3β,7α-diHCA and its dehydration product 3βH-7-DHCA.

9. Sum of intact 7αH,3O-CA and its dehydration product 3O-6-DHCA.

10. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

11. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

12. Identification based on comparison to 7α-isomer.

13. Identification based on comparison to 7α-isomer.

14. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

15. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

16. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

17. Authentic standard not available, annotation based on retention time, exact mass and MS^3 fragmentation.

Figure 1 (Griffiths)

(A)

(B)

(C)

(D)

(E)

(F)

Fig. 1. Concentration of 25-D$_3$ and cholesterol metabolites in serum. Box and whiskers plots showing the concentrations (ng/mL) of (A) 25-D$_3$, (B) 7α-HC, (C) 7α-HCO, (D) 7α,25-diHCO, (E) 26-HC, and (F) 3β-HCA in serum from ALS (n = 35) and PLS (n = 6) patients and healthy controls (n = 24). The bottom and top of the box are the first and third quartiles, and the band inside the box represents the median. The whiskers extend to the most extreme data points which are no more than 1.5 times the range between first and third quartile distant from the box. Points beyond that are plotted individually. Data for other sterols can be found in supplemental Table S1. Uni-variant t-tests were performed against the control group, *P < 0.05; **P < 0.01.

Figure 2 (Griffiths)

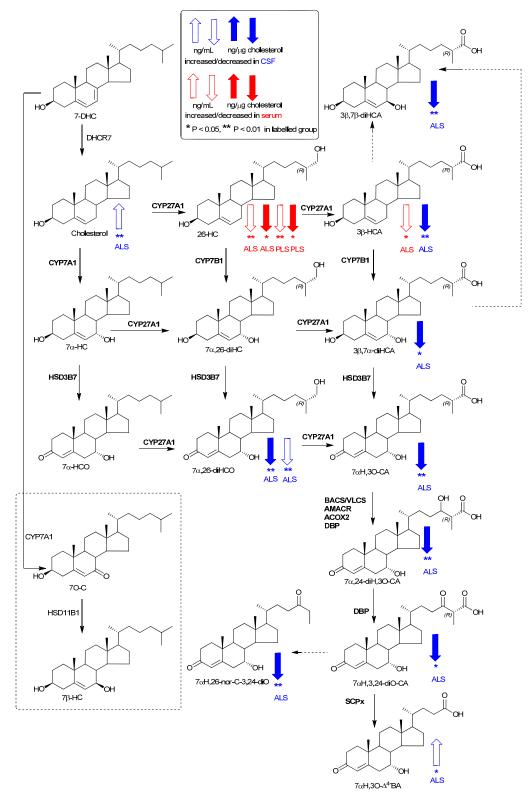Fig. 2. Pathways of cholesterol metabolism initiated by the enzymes cholesterol 25-hydroxylase (CH25H) and CYP46A1. Changes in sterols concentration in CSF and serum are indicated by blue and red arrows, respectively. The direction of change corresponds to the direction of the arrow. Enzymes catalysing the indicated reactions are shown where known. Uni-variant t-tests were performed against the control group, *P < 0.05; **P < 0.01.
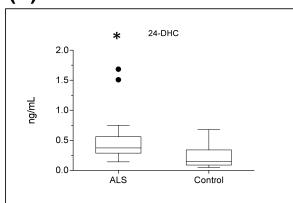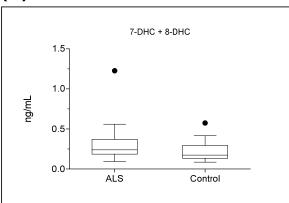
Figure 3 (Griffiths)

Fig. 3. Pathway of cholesterol metabolism initiated by the enzymes CYP7A1 and CYP27A1. Changes in sterols concentration in CSF and serum are indicated by blue and red arrows, respectively. The direction of change corresponds to the direction of the arrow. Enzymes catalysing the indicated reactions are shown where known. Enzyme abbreviations used are: ACOX2, acyl-CoA oxidase 2, branched chain; AMACR, alpha-methylacyl-CoA racemase; BACS, bile acyl-CoA synthetase; DBP, D-bifunctional protein or multifunctional enzyme type 2 (HSD17B4); SCPx, sterol carrier protein x; VLCS, very long chain acyl-CoA synthetase. Uni-variant t-tests were performed against the control group, *P < 0.05; **P < 0.01.

Figure 4 (Griffiths)
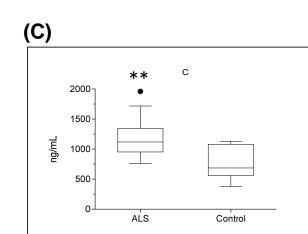
(A)

(B)

(C)

Fig. 4. Concentration of cholesterol and its precursors in CSF. Box and whisker plots showing concentrations (ng/mL) of (A) 24-DHC, (B) 7-DHC + 8-DHC, and (C) cholesterol, in CSF from ALS (n = 20) patients and health controls (n = 15). Box and whiskers are as described in the legend to Fig. 1. Data for other sterols can be found in supplemental Table S3. Uni-variant t-tests were performed against the control group, *P < 0.05; **P < 0.01.
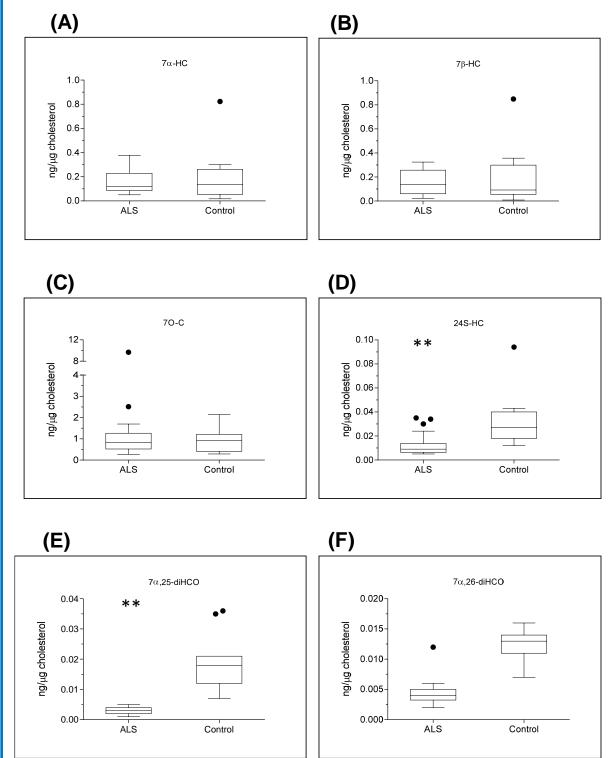
Figure 5 (Griffiths)

Fig. 5. Concentration of CYP7A1, CYP46A1 and CH25H pathway metabolites in CSF. Box and whisker plots showing concentrations (ng/μg cholesterol) of (A) 7α-HC, (B) 7β-HC, (C) 7O-C, (D) 24S-HC, (E) 7α,25-diHCO, and (F) 7α,26-diHCO in CSF from ALS (n = 20) patients and health controls (n = 15). Box and whiskers are as described in the legend to Fig. 1. Data for other sterols can be found in supplemental Table S4. Uni-variant t-tests were performed against the control group, *P < 0.05; **P < 0.01.

Figure 6 (Griffiths)

(A)

(B)

(C)

(D)

(E)

(F)

Fig. 6. Concentration of acidic pathway metabolites in CSF. Box and whisker plots showing concentrations (ng/µg cholesterol) of (A) 3β-HCA, (B) 3β,7α-diHCA, (C) 7αH,3O-CA, (D) 3β,7β-diHCA, (E) 7α,24-diH,3O-CA, and (F) 7αH,26-nor-C-3,24-diO in CSF from ALS (n = 20) patients and health controls (n = 15). Box and whiskers are as described in the legend to Fig. 1. Data for other sterols can be found in supplemental Table S4. Uni-variant t-tests were performed against the control group, *P < 0.05; **P < 0.01.
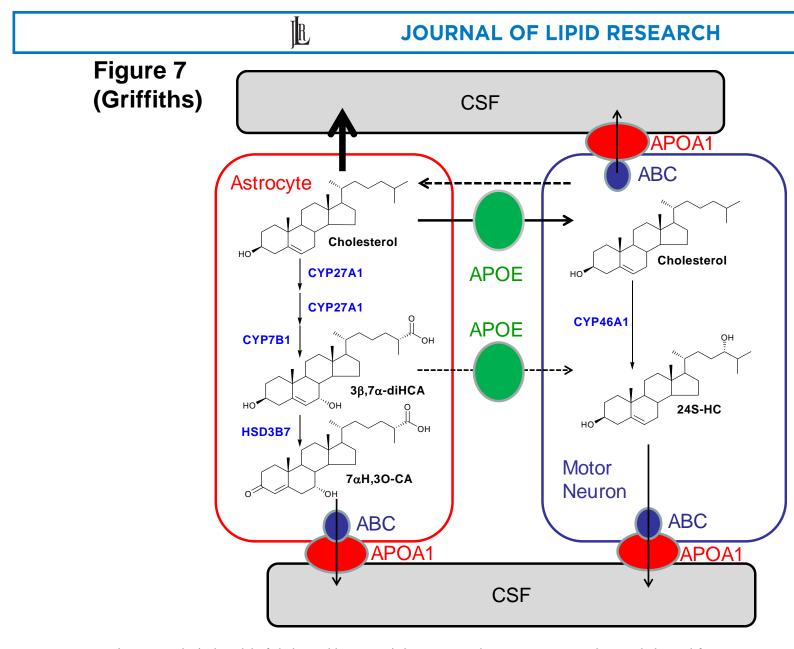
Figure 7 (Griffiths)

Fig. 7. Hypothetical model of cholesterol homeostasis in neurons and astrocytes. Neurons import cholesterol from astrocytes m
circle) (53). Neurons dispose of cholesterol by ATP-binding cassette (ABC) transporters (blue circle) and APOA1 (red circle),
to astrocytes via an unknown mechanism (broken arrow). Activation of LXRβ by oxysterols or cholestenoic acids leads to incr
increased sterol release. Impaired metabolism of cholesterol in astrocytes as suggested in the present study of ALS patients may
of astrocytes into the CSF and also into neurons. A reduction of metabolism of cholesterol to cholestenoic acids will result in
and lower amounts transported to neurons (broken arrow depicts unknown mechanism). A consequence will be increased cel
reduced anti-inflammatory signalling by LXRβ.