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### **Paper:**

Crick, P., Griffiths, W., Zhang, J., Beibel, M., Abdel-Khalik, J., Kuhle, J., Sailer, A. & Wang, Y. (2016). Reduced Plasma Levels of 25-Hydroxycholesterol and Increased Cerebrospinal Fluid Levels of Bile Acid Precursors in Multiple Sclerosis Patients. *Molecular Neurobiology*  
<http://dx.doi.org/10.1007/s12035-016-0281-9>

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# Reduced Plasma Levels of 25-Hydroxycholesterol and Increased Cerebrospinal Fluid Levels of Bile Acid Precursors in Multiple Sclerosis Patients

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Received: 12 July 2016 / Accepted: 31 October 2016

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**Abstract** Multiple sclerosis (MS) is an autoimmune, inflammatory disease of the central nervous system (CNS). We have measured the levels of over 20 non-esterified sterols in plasma and cerebrospinal fluid (CSF) from patients suffering from MS, inflammatory CNS disease, neurodegenerative disease and control patients. Analysis was performed following enzyme-assisted derivatisation by liquid chromatography–mass spectrometry (LC–MS) exploiting multistage fragmentation (*MS<sup>n</sup>*). We found increased concentrations of bile acid precursors in CSF from each of the disease states and that patients with inflammatory CNS disease classified as suspected autoimmune disease or of unknown aetiology also showed elevated concentrations of 25-hydroxycholesterol (25-HC,  $P < 0.05$ ) in CSF. Cholesterol concentrations in CSF were not changed except for patients diagnosed with

amyotrophic lateral sclerosis ( $P < 0.01$ ) or pathogen-based infections of the CNS ( $P < 0.05$ ) where they were elevated. In plasma, we found that 25-HC ( $P < 0.01$ ), (25R)26-hydroxycholesterol ((25R)26-HC,  $P < 0.05$ ) and 7 $\alpha$ -hydroxy-3-oxocholest-4-enoic acid (7 $\alpha$ H,3O-CA,  $P < 0.05$ ) were reduced in relapsing-remitting MS (RRMS) patients compared to controls. The pattern of reduced plasma levels of 25-HC, (25R)26-HC and 7 $\alpha$ H,3O-CA was unique to RRMS. In summary, in plasma, we find that the concentration of 25-HC in RRMS patients is significantly lower than in controls. This is consistent with the hypothesis that a lower propensity of macrophages to synthesise 25-HC will result in reduced negative feedback by 25-HC on IL-1 family cytokine production and exacerbated MS. In CSF, we find that the dominating metabolites reflect the acidic pathway of bile acid biosynthesis and the elevated levels of these in CNS disease is likely to reflect cholesterol release as a result of demyelination or neuronal death. 25-HC is elevated in patients with inflammatory CNS disease probably as a consequence of up-regulation of the type 1 interferon-stimulated gene *cholesterol 25-hydroxylase* in macrophages.

**Electronic supplementary material** The online version of this article (doi:10.1007/s12035-016-0281-9) contains supplementary material, which is available to authorized users.

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**Keywords** Sterol · Oxysterol · Bile acid · CNS · Inflammation · HPLC · MS

## Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS). A large number of genetic variants, together with several putative environmental agents, including serum vitamins D levels, Epstein–Barr virus infection and a history of smoking, determine MS susceptibility [1, 2]. The disease in most cases follows a relapsing–remitting (RR) course where acute autoimmune attacks

against the CNS are followed by recovery. Many patients with RRMS go on to develop secondary progressive MS, characterised by irreversible neurological disability. Immunological and pathological studies have provided evidence supporting an important role played by the immune system in driving the abnormal demyelinating process seen in MS patients [3, 4]. Neuronal degeneration is also a key in MS pathogenesis and is present already in early disease stages and is especially evident in the progressive phase of the disease, when brain atrophy and irreversible disability are more prominent [4, 5]. Type 1 interferon (IFN) is used in the treatment of MS, it has a suppressive effect on immunity [6]. IFN-stimulated genes include *cholesterol 25-hydroxylase (CH25H)* which is up-regulated in macrophages upon bacterial or virus infection [7–10]. Recently, several disease-modifying drugs (DMDs) in addition to the first generation of injectable DMDs (IFN and glatiramer acetate) have been developed and licensed. These include natalizumab, fingolimod, dimethyl fumarate, teriflunomide and alemtuzumab.

Perturbation of sterol and cholesterol pathways has recently been linked to various immune disorders [11, 12]. Oxysterols, oxidised metabolites of cholesterol or its precursors, are key mediators of these pathways. As well as being essential metabolites controlling cholesterol levels and leading to the production of bile acids, oxysterols have been shown to modulate the immune system. They, and their downstream metabolites, are ligands for nuclear hormone receptors such as the liver X receptors (LXRs), the farnesoid X receptor, the pregnane X receptor, the RAR-related orphan receptor  $\gamma$  (RORc2) [13–16], they modulate transcription in macrophages [17], and RORc2 activation plays a central role in the differentiation of  $T_H17$  cells [18]. Furthermore, oxysterols can activate a G protein-coupled receptor called Epstein–Barr virus-induced gene 2 (EBI2, GPR183) and oxysterol gradients guide migration of EBI2 expressing immune cells [19, 20], many of which have been implicated in shaping the adapted and innate immune response. We hypothesised that oxysterol concentrations vary under pathophysiological conditions and set out to determine using liquid chromatography–mass spectrometry (LC–MS) oxysterol levels in plasma and cerebrospinal fluid (CSF) from patients suffering from MS, both RRMS and clinically isolated syndrome (CIS), and from symptomatic control patients (CP). Note, we use italics to differentiate the abbreviation for mass spectrometry (MS), from that for multiple sclerosis (MS). In addition, samples from patients with neurodegenerative disease i.e. Alzheimer’s disease (AD) or Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and inflammatory CNS disease i.e. suspected autoimmune disease or of unknown aetiology (SA/UA) and pathogen-based infection (PBI), were analysed. The CSF data for the CP group has been published elsewhere [21].

## Materials and Methods

### Patients and Controls

Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and the study was approved by the Common Institutional Review Board of the Cantons of Basel, Switzerland.

Samples were stratified into the following groups: (1) CIS, ( $n = 16$ ); (2) RRMS, ( $n = 17$ ); (3) CP ( $n = 18$ ), i.e. patients with neurological symptoms, but no objective clinical or paraclinical findings to define a specific neurological disease at the time of sampling (CSF negative for oligoclonal bands, normal blood brain barrier function, and normal cell count); (4) inflammatory CNS disease subdivided into (4.1) SA/UA ( $n = 10$ ); and (4.2) PBI ( $n = 9$ ); and (5) neurodegenerative diseases made up of (5.1) AD and PD ( $n = 9$ ); and (5.2) ALS ( $n = 11$ ). The Table shown in Online Resource 1 summarises patient information.

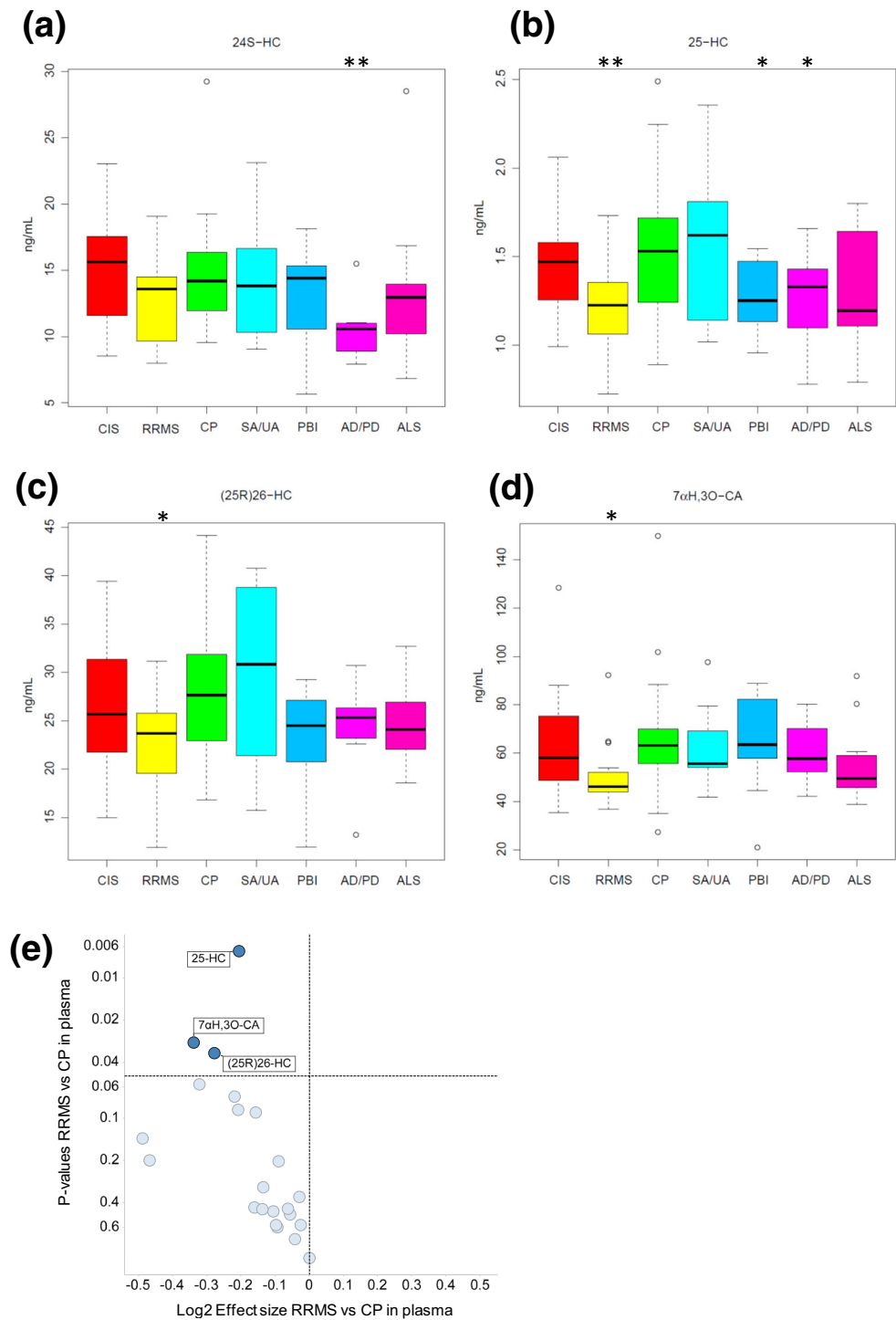
### Lipid Extraction and LC–MS

Non-esterified sterols in plasma were assayed by LC–MS exploiting enzyme-assisted derivatisation utilising the Girard P (GP) reagent as illustrated in the Fig. in Online Resource 2. The method is fully described in [22]. For CSF analysis, the only modifications made to the published protocol for plasma were that the volume of CSF used was 250  $\mu$ L, while that for plasma was 100  $\mu$ L; the concentrations of internal standards were 16 ng/mL of 24R/S-[25,26,26,26,27,27,27- $^2$ H $_7$ ]hydroxycholesterol, 1.6 ng/mL of 7 $\alpha$ ,25-[26,26,26,27,27,27- $^2$ H $_6$ ]dihydroxycholesterol, 16 ng/mL of 22R-[25,26,26,26,27,27,27- $^2$ H $_7$ ]hydroxycholesterol-4-en-3-one and 16  $\mu$ g/mL of [25,26,26,26,27,27,27- $^2$ H $_7$ ]cholesterol. The size of the final C $_{18}$  column used for CSF was 50 mg while that used for plasma was 200 mg. Exact details are given in Online Resource 3.

### Statistical Analysis

An ANOVA test was run for each sterol on linear and logarithmic scales. The log scale used a transformation  $\log_2(+1)$  to avoid issues with zero and small numbers. Uni-variant  $t$  tests were performed against the CP group,  $*P < 0.05$ ;  $**P < 0.01$ . Concentrations given in the text are mean  $\pm$  standard deviation (SD). The boxplots in Figs. 1 and 2 and the Figs. in Online Resources 4 and 5 were generated with default parameters in R version 3.02. The bottom and top of the central box are the first and third quartiles, and the band inside the box is the median. The whiskers extend to the most extreme data points which are no more than 1.5 times the range between the first and third quartile distant from the box. Points beyond that are plotted individually. Pair-wise correlations between CSF or

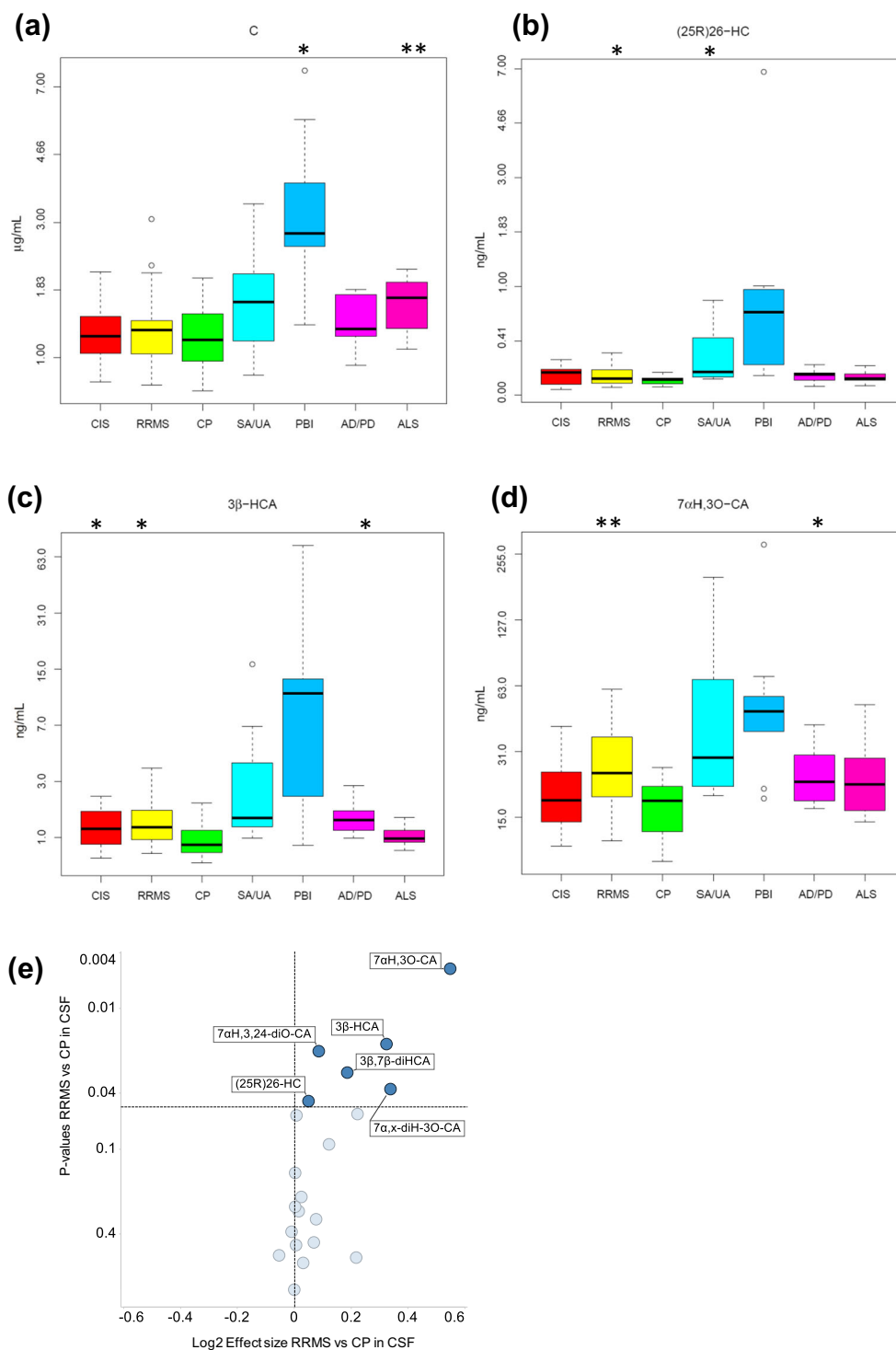
**Fig. 1** Effect of CNS disease on sterol concentrations in plasma. *Box and whiskers plots* showing the concentrations (ng/mL) of **a** 24S-HC, **b** 25-HC, **c** (25R)26-HC, and **d** 7 $\alpha$ H,3O-CA in plasma from CIS ( $n = 16$ ), RRMS ( $n = 17$ ), CP ( $n = 18$ ), SA/UA ( $n = 10$ ), PBI ( $n = 9$ ), AD/PD ( $n = 9$ ) and ALS ( $n = 11$ ) patients. Uni-variant  $t$  tests were performed against the CP group,  $*P < 0.05$ ;  $**P < 0.01$ . **e** Volcano plot for plasma data showing the  $P$  value versus base2 logarithm of fold change for the RRMS group against CP



plasma levels and specific analyte were performed by R version 3.02.  $P$  values for the significance of the correlations are listed in the Tables in Online Resources 6 and 7, respectively. The  $P$  values that are below  $0.05/((21*20)/2) = 0.000238$  for CSF and below  $0.05/((22*21)/2) = 0.000216$  for plasma are highlighted in the Online Resource Tables, these are significant after a Bonferroni correction at 5 %.

The volcano plots of statistical significance ( $P$  values) against log2 fold change between RRMS and CP shown in Figs. 1e and 2e, demonstrating the most significantly differentially regulated metabolites, were generated with TIBCO Spotfire (TIBCO Software) using log2(+1) transformation. The  $t$  test was made using the modification by Welch that accommodates for different variances in the two groups.

**Fig. 2** Effect of CNS disease on sterol concentrations in CSF. *Box and whiskers plots* showing the concentrations of **a** cholesterol, **b** (25R)26-HC, **c** 3 $\beta$ -HCA and **d** 7 $\alpha$ H,3O-CA in CSF from CIS ( $n = 16$ ), RRMS ( $n = 17$ ), CP ( $n = 18$ ), SA/UA ( $n = 10$ ), PBI ( $n = 9$ ), AD/PD ( $n = 9$ ) and ALS ( $n = 11$ ) patients. Cholesterol concentration is in microgram per milliliter other analyte concentrations are in nanogram per milliliter. To facilitate visualisation, the  $y$ -axis is on a  $\log_2$  scale. Uni-variant  $t$  tests were performed against the CP group. \* $P < 0.05$ ; \*\* $P < 0.01$ . **e** Volcano plot for CSF data showing the  $P$  value versus base2 logarithm of fold change for the RRMS group against CP



## Results

### Plasma

Shown in Fig. 1a–d and in the Fig. in Online Resource 4 are box and whisker plots showing the concentrations of non-esterified oxysterols, 25-hydroxyvitamin D<sub>3</sub> (25-D<sub>3</sub>), cholestenic and choleonic acids in plasma. Table 1 lists the

sterols analysed and the Table in Online Resource 8 gives the measured concentrations for each patient group. In the RRMS samples, the concentration of 25-hydroxycholesterol (25-HC) is reduced significantly compared to CP ( $1.19 \pm 0.26$  ng/mL cf.  $1.54 \pm 0.42$  ng/mL, mean  $\pm$  SD,  $P < 0.01$ ). We also see a reduction in the concentration of (25R)26-hydroxycholesterol ((25R)26-HC,  $22.63 \pm 5.03$  ng/mL cf.  $27.97 \pm 7.89$  ng/mL,  $P < 0.05$ ) and 7 $\alpha$ -hydroxy-3-oxocholest-4-enoic acid

**Table 1** Oxysterols, cholestenic and cholenic acids and vitamin D<sub>3</sub> metabolites analysed by LC–MS in the present study. Concentrations measured in plasma and CSF are given in Online Resources 8 and 9, respectively

Sterol systematic name (common name)	Lipid maps ID	Abbreviation	Code
9,10-Secocholesta-5Z,7E,10-trien-3 $\beta$ ,25-diol (25-Hydroxyvitamin D <sub>3</sub> )	LMST03020246	25-D <sub>3</sub>	C_1
Cholest-5-en-3 $\beta$ -ol (cholesterol)	LMST01010001	C	C_2
Cholest-4-ene-3 $\beta$ ,6-diol or Cholest-5-ene-3 $\beta$ ,6-diol (6-hydroxycholesterol)	–	6-HC	C_3
Cholest-5-ene-3 $\beta$ ,7 $\alpha$ -diol (7 $\alpha$ -hydroxycholesterol)	LMST01010013	7 $\alpha$ -HC	C_4
7 $\alpha$ -Hydroxycholest-4-en-3-one	LMST04030123	7 $\alpha$ -HCO	C_5
Cholest-5-ene-3 $\beta$ ,7 $\beta$ -diol (7 $\beta$ -hydroxycholesterol)	LMST01010047	7 $\beta$ -HC	C_6
3 $\beta$ -Hydroxycholest-5-en-7-one (7-oxocholesterol)	LMST01010049	7O-C	C_7
Cholest-5-ene-3 $\beta$ ,24S-diol (24S-hydroxycholesterol)	LMST01010019	24S-HC	C_8
Cholest-5-ene-3 $\beta$ ,25-diol (25-hydroxycholesterol)	LMST01010018	25-HC	C_9
Cholest-5-ene-3 $\beta$ , (25R)26-diol ((25R)26-hydroxycholesterol)	LMST01010088	(25R)26-HC	C_10
Cholest-5-ene-3 $\beta$ ,7 $\alpha$ ,25-triol (7 $\alpha$ ,25-Dihydroxycholesterol)	LMST04030166	7 $\alpha$ ,25-diHC	C_11
7 $\alpha$ ,25-Dihydroxycholest-4-en-3-one	LMST04030107	7 $\alpha$ ,25-diHCO	C_12
Cholest-5-ene-3 $\beta$ ,7 $\alpha$ , (25R)26-triol (7 $\alpha$ , (25R)26-dihydroxycholesterol)	LMST04030081	7 $\alpha$ , (25R)26-diHC	C_13
7 $\alpha$ , (25R)26-Dihydroxycholest-4-en-3-one	LMST04030157	7 $\alpha$ , (25R)26-diHCO	C_14
3 $\beta$ -Hydroxycholest-5-enoic acid	LMST04030072	3 $\beta$ -HCA	C_15
3-Oxocholest-4-enoic acid	LMST04030217	3O-CA	C_16
3 $\beta$ ,7 $\beta$ -Dihydroxycholest-5-enoic acid	–	3 $\beta$ ,7 $\beta$ -diHCA	C_17
3 $\beta$ ,7 $\alpha$ -Dihydroxycholest-5-enoic acid	LMST04030148	3 $\beta$ ,7 $\alpha$ -diHCA	C_18
7 $\alpha$ -Hydroxy-3-oxocholest-4-enoic acid	LMST04030149	7 $\alpha$ H,3O-CA	C_19
7 $\alpha$ ,x-Dihydroxy-3-oxocholest-4-enoic acid	–	7 $\alpha$ ,x-diH,3O-CA	C_20
7 $\alpha$ ,y-Dihydroxy-3-oxocholest-4-enoic acid	–	7 $\alpha$ ,y-diH,3O-CA	C_21
7 $\alpha$ -Hydroxy-3,24-bisoxocholest-4-enoic acid	–	7 $\alpha$ H,3,24-diO-CA	C_22
7 $\alpha$ -Hydroxy-26-nor-cholest-4-ene-3,24-dione	–	7 $\alpha$ H,26-nor-C-3,24-diO	C_23
3 $\beta$ -Hydroxychol-5-enoic acid	LMST04010201	3 $\beta$ H- $\Delta^5$ -BA	C_24
3 $\beta$ ,7 $\alpha$ -Dihydroxychol-5-enoic acid	LMST04010217	3 $\beta$ ,7 $\alpha$ -diH- $\Delta^5$ -BA	C_25
7 $\alpha$ -Hydroxy-3-oxochol-4-enoic acid	LMST04010239	7 $\alpha$ H,3O- $\Delta^4$ -BA	C_26

Note that we use the systematic nomenclature where addition of a hydroxy group to the terminal side chain of cholesterol leading to R stereochemistry at C-25 gives the compound (25R)26-hydroxycholesterol. In much of the literature, this compound is known by the non-systematic name 27-hydroxycholesterol

(7 $\alpha$ H,3O-CA,  $50.60 \pm 13.33$  ng/mL cf.  $66.88 \pm 26.93$  ng/mL  $P < 0.05$ ) in RRMS patients. It is important to note that 25-HC is generated by macrophages [7, 9, 23], while the latter two compounds are synthesised by multiple different cell types, making their exact origin in plasma difficult to assess [24]. The levels of the 25-HC metabolites 7 $\alpha$ ,25-dihydroxycholesterol (7 $\alpha$ ,25-diHC) and 7 $\alpha$ ,25-dihydroxycholest-4-en-3-one (7 $\alpha$ ,25-diHCO) in RRMS plasma do not differ from CP values ( $0.22 \pm 0.08$  ng/mL and  $1.06 \pm 0.33$  ng/mL, respectively, Fig. 3). This data suggests that the fall in 25-HC concentration in RRMS patients is a consequence of reduced production of 25-HC arising from either reduced transcription/translation of *CH25H* or reduced activity of the enzyme and that RRMS patients have a reduced capacity to produce 25-HC.

In agreement with some, but not all, earlier studies, the level of 24S-hydroxycholesterol (24S-HC) is reduced in plasma from the group of patients with either AD or PD compared

to CP ( $10.51 \pm 2.18$  ng/mL cf.  $14.99 \pm 4.57$  ng/mL,  $P < 0.01$ ) [25, 26]. The level of 25-HC is also reduced in plasma of AD/PD patients ( $1.26 \pm 0.28$  ng/mL,  $P < 0.05$ ), as it is in patients diagnosed with PBI ( $1.28 \pm 0.21$  ng/mL,  $P < 0.05$ ), compared to CP ( $1.54 \pm 0.42$  ng/mL).

### CSF

It is generally considered that in healthy individuals CSF levels of (25R)26-HC correlate with levels of this oxysterol in the circulation [27]. However, this is not necessarily the case with (25R)26-HC in CSF from patients with neurodegenerative diseases [28]. In our study, we find patients with RRMS, in contrast to our observations in plasma, show an increase in the concentration of (25R)26-HC in CSF ( $0.14 \pm 0.07$  ng/mL,  $P < 0.05$ ) compared to CP ( $0.10 \pm 0.03$  ng/mL, Fig. 2, (see also Online Resources 5 and 9). (25R)26-HC can be metabolised in the CNS to

$7\alpha\text{H},3\text{O-CA}$  (Fig. 4) [29, 30]. We find that the concentration of this acid is elevated in RRMS CSF ( $27.59 \pm 12.93$  ng/mL,  $P < 0.01$ ) compared to CP ( $17.40 \pm 4.63$  ng/mL), as is the level of its precursor  $3\beta$ -hydroxycholest-5-enoic acid ( $3\beta$ -HCA,  $1.52 \pm 0.85$  ng/mL cf.  $0.96 \pm 0.42$ ,  $P < 0.05$ ). The intermediary metabolite between these two acids,  $3\beta,7\alpha$ -dihydroxycholest-5-enoic acid ( $3\beta,7\alpha$ -diHCA), is also elevated ( $3.71 \pm 4.07$  ng/mL cf.  $2.12 \pm 1.65$  ng/mL) but not to significance. MS is a demyelinating disease and it likely that cholesterol is released in the CNS is metabolised through (25R)26-HC and  $3\beta$ -HCA to  $7\alpha\text{H},3\text{O-CA}$ , although the actual concentration of non-esterified cholesterol in CSF does not differ from controls ( $1.24 \pm 0.33$   $\mu\text{g/mL}$ ).  $3\beta,7\beta$ -Dihydroxycholest-5-enoic acid ( $3\beta,7\beta$ -diHCA) which may be a metabolic product of  $3\beta$ -HCA,  $3\beta,7\alpha$ -diHCA or 7-oxocholesterol (7O-C) is also elevated in CSF from RRMS patients ( $0.62 \pm 0.35$  ng/mL cf.  $0.40 \pm 0.19$  ng/mL,  $P < 0.05$ ).

The CSF from the ALS group shows differences in cholesterol metabolite concentrations compared to controls and also in the concentration of cholesterol itself ( $1.66 \pm 0.36$   $\mu\text{g/mL}$  cf.  $1.24 \pm 0.33$  ng/mL,  $P < 0.01$ ). The concentration of  $7\alpha,(25\text{R})26$ -dihydroxycholest-4-en-3-one ( $7\alpha,(25\text{R})26$ -diHCO,  $0.03 \pm 0.01$  ng/mL, cf.  $0.02 \pm 0.01$  ng/mL,  $P < 0.01$ ) is increased as is its downstream metabolite  $7\alpha\text{H},3\text{O-CA}$ , although not quite to significance ( $24.45 \pm 11.16$  ng/mL, cf.  $17.40 \pm 4.63$  ng/mL,  $P = 0.07$ ). Other neurodegenerative diseases including AD and PD are also believed to result in increased cholesterol release in brain as neurons die [28]. Björkhem and colleagues have found that patients with PD or AD can have higher CSF levels of (25R)26-HC than the controls [28, 31, 32]. We find increased concentrations of metabolites of (25R)26-HC but not of the oxysterol itself i.e. concentrations of  $3\beta$ -HCA ( $1.57 \pm 0.55$  ng/mL cf.  $0.96 \pm 0.42$  ng/mL,  $P < 0.05$ ),  $7\alpha\text{H},3\text{O-CA}$  ( $25.53 \pm 9.52$  ng/mL cf.  $17.40 \pm 4.63$  ng/mL,  $P < 0.05$ ) and  $3\beta,7\beta$ -diHCA ( $0.64 \pm 0.16$  ng/mL, cf.  $0.40 \pm 0.19$  ng/mL,  $P < 0.01$ ) are elevated in this disease group.

Patients diagnosed with PBI show an increase in concentration of  $7\alpha,(25\text{R})26$ -diHCO ( $0.05 \pm 0.03$  ng/mL cf.  $0.02 \pm 0.01$  ng/mL,  $P < 0.05$ ) in CSF while those diagnosed with SA/UA show increased concentrations of (25R)26-HC ( $0.30 \pm 0.26$  ng/mL cf.  $0.10 \pm 0.03$  ng/mL,  $P < 0.05$ ),  $7\alpha,(25\text{R})26$ -dihydroxycholesterol ( $7\alpha,(25\text{R})26$ -diHC,  $0.01 \pm 0.01$  ng/mL cf.  $0.00 \pm 0.00$  ng/mL,  $P < 0.05$ ) and  $7\alpha,(25\text{R})26$ -diHCO ( $0.04 \pm 0.02$  ng/mL cf.  $0.02 \pm 0.01$  ng/mL,  $P = 0.01$ ).

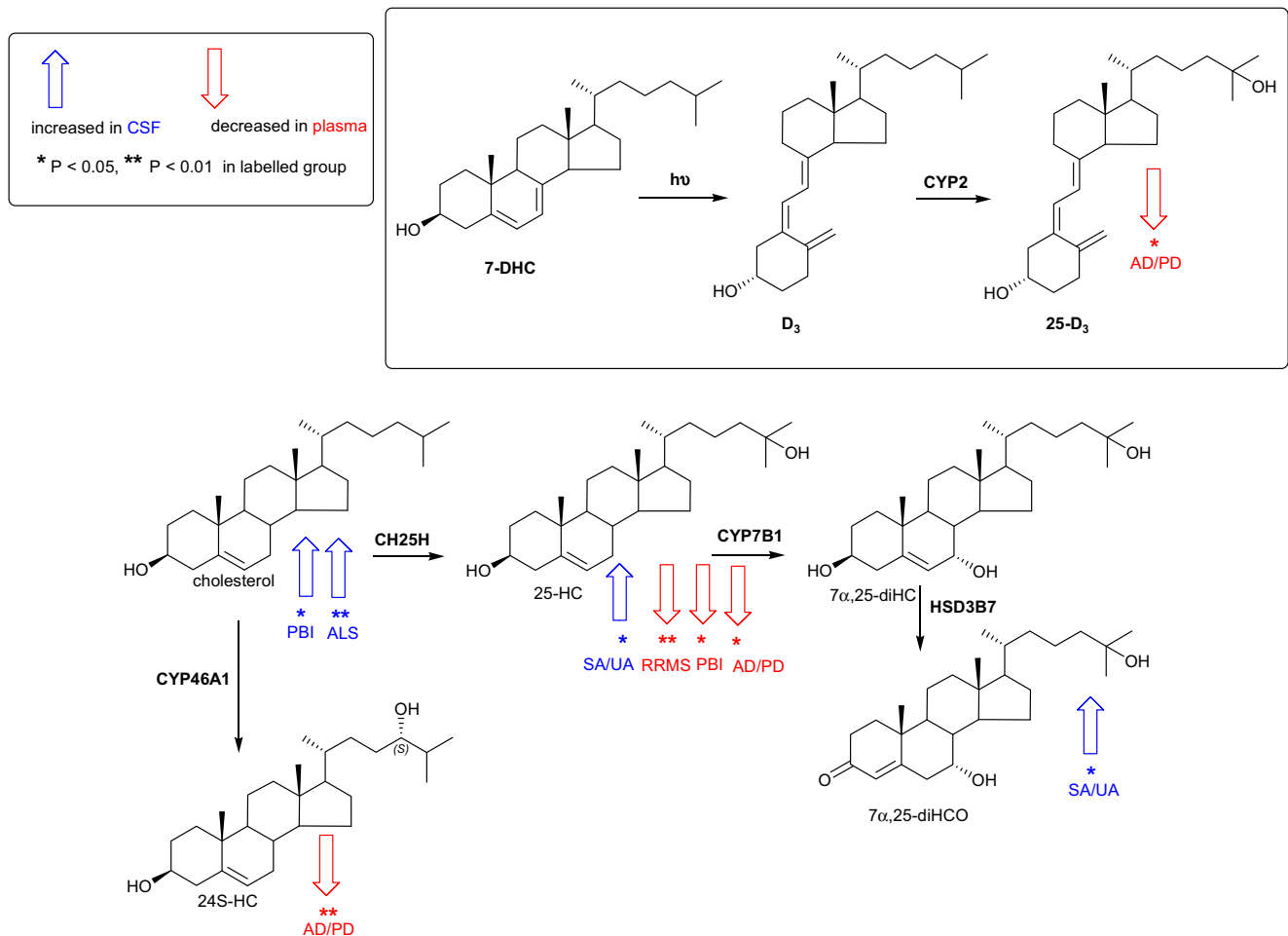
The limit of quantification of our analytical method in CSF for most analytes is 0.01 ng/mL (10:1, signal to noise); however, for 25-HC, it is somewhat higher at 0.03 ng/mL, although detection can be made at 0.01 ng/mL. Only in the patient group of SA/UA ( $0.06 \pm 0.04$  ng/mL, cf.  $0.03 \pm 0.02$  ng/mL,  $P < 0.05$ ) did any of the disease states show any statistical differences in the level of 25-HC in CSF;

this is also true for the 25-HC metabolite  $7\alpha,25$ -diHCO ( $0.07 \pm 0.03$  ng/mL cf.  $0.04 \pm 0.02$  ng/mL,  $P < 0.05$ ) (Fig. 3). The level of non-esterified cholesterol in control CSF is  $1.24 \pm 0.33$   $\mu\text{g/mL}$ . It is found to be elevated in the PBI group ( $3.44 \pm 2.05$   $\mu\text{g/mL}$   $P < 0.05$ ).

## Discussion

### 25-HC Is Reduced in RRMS Plasma

In the current study, we have measured the levels of non-esterified sterols, oxysterols, 25-D<sub>3</sub> and of cholestenic and cholenoic acids in plasma and CSF of patients with MS, neurodegenerative and inflammatory CNS disease. We find that the level of 25-HC is significantly changed ( $P < 0.01$ ) in plasma from patients with RRMS, falling from  $1.54 \pm 0.42$  ng/mL in CP to  $1.19 \pm 0.26$  ng/mL in RRMS plasma (Figs. 1b, e and 3). This suggests reduced transcription/translation of the gene *CH25H* in macrophages, reduced activity of the CH25H enzyme, or alternatively, enhanced clearance or metabolism of 25-HC in RRMS patients. The latter possibility is unlikely as there was no increase in concentration of downstream metabolites in plasma. 25-HC levels were also reduced, but to a lesser extent ( $P < 0.05$ ), in plasma from patients with PBI and AD or PD. Non-esterified oxysterols are present in CSF at pg/mL levels [21], and in control subjects 25-HC is at our limit of quantification (0.03 ng/mL) and was not found to change in any of the patient groups except those with SA/UA where the level increased to  $0.06 \pm 0.04$  ng/mL ( $P < 0.05$ ). The plasma data for RRMS patients, indicating a reduced capacity of macrophages to synthesise 25-HC, is in line with the recent study by Reboldi et al. which shows that in mouse macrophages 25-HC is a mediator of negative feedback towards interleukin 1 (IL-1) family cytokine production and inflammasome activity, through binding to INSIG (insulin-induced gene) and antagonising the sterol response element-binding protein-2 (SREBP-2) driven mevalonate pathway, thereby, reducing *Il1b* transcription and repressing IL-1-activating inflammasomes [33]. Thus, reduced amounts of 25-HC lead to less negative feedback towards IL-1 family cytokine production in the macrophage, with the consequent enhancement of inflammation. In keeping with these actions of 25-HC, *Ch25h* knockout mice show exacerbated experimental autoimmune encephalomyelitis (EAE), an IL-17-driven inflammatory disease model of MS, and increased susceptibility to septic shock. *Ch25h*-deficient macrophages produce more proinflammatory IL-17A<sup>+</sup> T cells following lipopolysaccharide (LPS) stimulation and cytokines that cooperate with transforming growth factor- $\beta$  (TGF $\beta$ ) to induce T<sub>H</sub>17 cells include IL-1 $\beta$ . Notably, after LPS stimulation *Ch25h*-deficient macrophages overproduce *Il1b* [33].



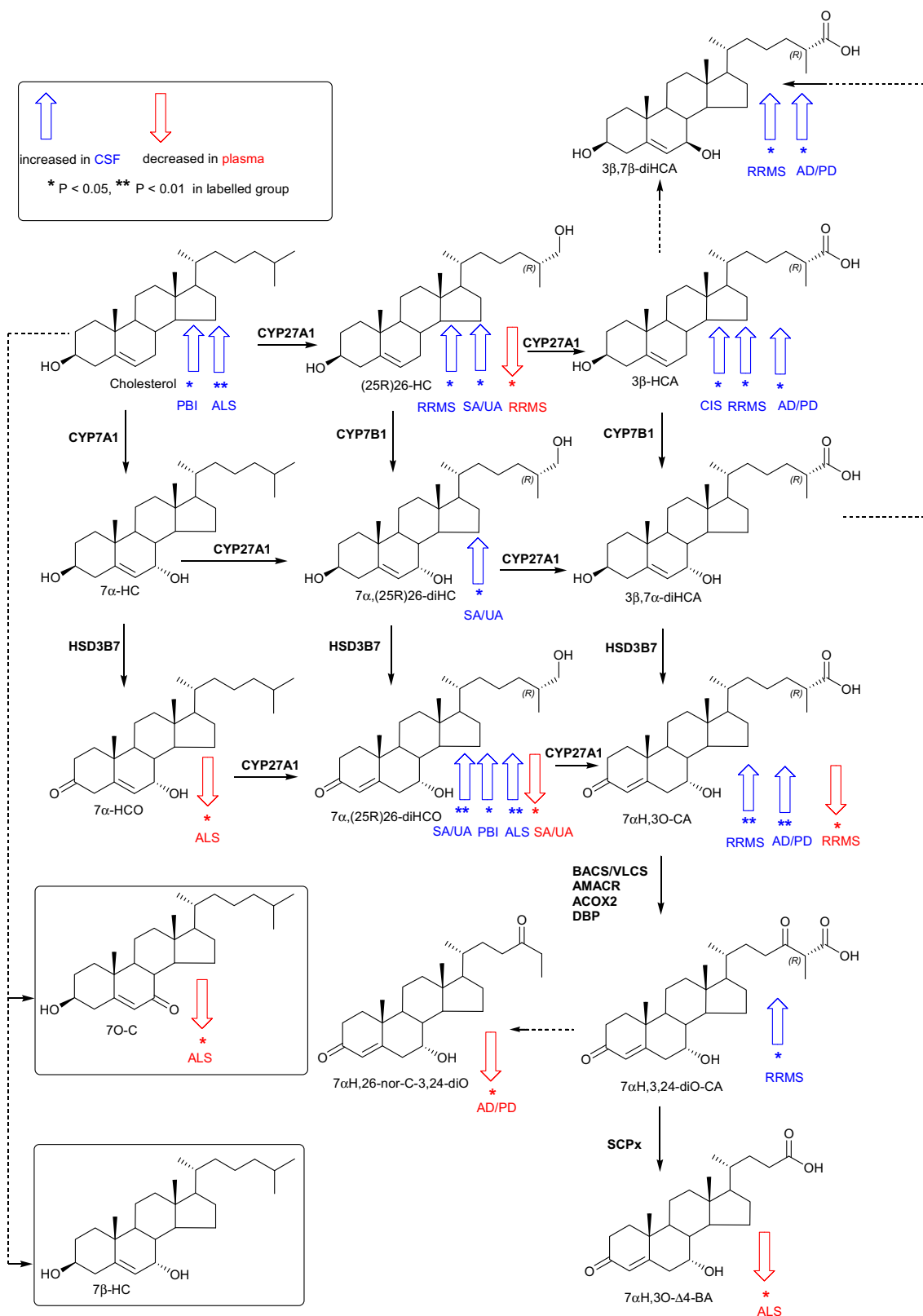
**Fig. 3** Sterol metabolism via the cholesterol 24S- and 25-hydroxylase pathways in CNS. Changes in sterol concentrations in CSF and plasma are indicated by blue and red arrows, respectively. The direction of

change corresponds to the direction of the arrow. Enzyme abbreviations used are *CH25H* cholesterol 25-hydroxylase, *CYP* cytochrome P450, *HSD* hydroxysteroid dehydrogenase

The mechanism of action by which reduced SREBP processing correlates with a reduction in *Il1b* transcription is not known. Reboldi et al. suggested that this may be through reducing the cellular content of sterols generated via the mevalonate pathway [33]. The results of Reboldi et al. are in contrast to those of Chalmin et al. who reported that inactivation of the *Ch25h* gene significantly attenuates EAE by limiting trafficking of pathogenic CD4+ T lymphocyte to the CNS [34]. Clearly, more work is needed to reconcile these divergent results in the literature. Although (25R)26-HC and 7 $\alpha$ H,3O-CA were, like 25-HC, also found to be reduced ( $P < 0.05$ ) in RRMS plasma (Fig. 1), the latter is not known to bind to INSIG, while neither sterol is formed by IFN-activated macrophages to any significant extent [8]. In contrast, 25-HC is formed by macrophages in response to IFN [7, 9, 35] and macrophages are important effector cells involved in the pathogenesis of demyelination in MS. 25-HC is also an agonist to the LXRs [36], as is (25R)26-HC but to a lesser extent and not at all in neuronal cells [21]. Besides being regulators of lipid metabolism, LXRs have also been found to modulate immune

and inflammatory responses in macrophages [37]. Thus, reduced LXR activation, as a consequence of diminished 25-HC, may also explain an enhanced inflammatory response in RRMS. The involvement of LXR in the aetiology of MS is further supported by the recent report by Meffre et al. showing that LXRs are involved in the myelination and remyelination processes in oligodendrocytes [38]. In fact, LXR $\alpha$  or LXR $\beta$  when activated by 25-HC stimulates myelin gene expression at the promoter, mRNA and protein levels, directly implicating LXRs in the transcriptional control of myelin gene expression [38]. In addition, Wang et al. have found an LXR $\alpha$  mutation, in two multi-incident families presenting with severe and progressive MS disease, that disrupts LXR $\alpha$  heterodimerisation and transcriptional activation of target genes, further linking LXR and its agonists with MS pathogenesis [39]. In summary, a reduced capacity of macrophages to generate 25-HC may result in enhanced activity of the mevalonate pathway leading to over production of *Il1b*, and to reduced LXR activation which will both lead to enhanced inflammatory activity of macrophages recruited to the CNS in





**Fig. 4** Sterol metabolism via the bile acid biosynthesis pathways in CNS. The acidic pathway starts with (25R)26-hydroxylation of cholesterol by CYP27A1, the neutral pathway with 7 $\alpha$ -hydroxylation of cholesterol by CYP7A1. Changes in sterol concentration in CSF and plasma are indicated by blue and red arrows, respectively. The direction of change

corresponds to the direction of the arrow. 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

MS. It might be expected that similar to the situation in plasma, 25-HC concentrations are reduced in CSF from RRMS patients; however, 25-HC concentrations in control and RRMS patients were near the limit of quantification making statistical evaluation difficult.

### Demyelination Leads to Activation of Bile Acid Biosynthesis

In contrast to plasma, there is a significant elevation in (25R)26-HC ( $P < 0.05$ ) and of its metabolites  $3\beta$ -HCA ( $P < 0.05$ ) and  $7\alpha$ H,3O-CA ( $P < 0.01$ ) in CSF of RRMS patients (Fig. 2). These cholesterol metabolites represent early members of the “acidic pathway” of bile acid biosynthesis [40] which is known to be operative in the CNS [29, 30]. A latter member of this pathway,  $7\alpha$ -hydroxy-3,24-*bis*-oxocholest-4-enoic acid ( $7\alpha$ H,3,24-diO-CA), formed in the peroxisome as a thioester is also found to be elevated in RRMS CSF (Fig. 4). MS is a demyelinating disease with the consequent release of non-esterified cholesterol in the CNS. Cholesterol is metabolised in the CNS in astrocytes through cytochrome P450 (CYP) 27A1 catalysed oxidation to (25R)26-HC and subsequently to  $3\beta$ -HCA and then on to  $3\beta,7\alpha$ -diHCA and  $7\alpha$ H,3O-CA by the consecutive action of CYP7B1 and hydroxysteroid dehydrogenase (HSD) 3B7 enzymes (Fig. 4) [41]. An increase in the availability in CNS of non-esterified cholesterol, the primary CYP27A1 substrate, is the likely explanation for the elevated activity of this pathway. Patients with CIS, which is MS resulting from a single episode of demyelination in one area of the CNS, also show an increase in the CYP27A1 product  $3\beta$ -HCA ( $P < 0.05$ ) but not its downstream metabolites. Interestingly, both  $3\beta$ -HCA and  $3\beta,7\beta$ -diHCA have been shown to be neurotoxic [21].

Patients with neurodegenerative disease including AD and PD, or ALS also show an elevation in the metabolites of the “acidic pathway” in CSF. The cholesterol metabolites  $3\beta$ -HCA ( $P < 0.05$ ) and  $7\alpha$ H,3O-CA ( $P < 0.01$ ) of the “acidic pathway” are elevated in the AD and PD group, while in the ALS group  $7\alpha$ , (25R)26-diHCO ( $P < 0.01$ ) is increased (Fig. 4). There are very few other studies of cholestenic acids in CSF; however, Saeed et al. found that levels of  $7\alpha$ H,3O-CA were similar in AD patients and controls [42]. Brown et al. have found that CYP27A1 expression increases in oligodendrocytes in AD; this may provide an additional route to  $3\beta$ -HCA from cholesterol released by dying neurons in this disease [43].

Patients with inflammatory CNS disease include those diagnosed with SA/UA and those diagnosed with PBI. The patients with SA/UA show elevated levels of 25-HC in CSF ( $P < 0.05$ , Fig. 3) but not in the circulation. Notably, the metabolite of 25-HC,  $7\alpha$ ,25-diHCO is also elevated in CSF of these patients ( $P < 0.05$ ) as are (25R)26-HC ( $P < 0.05$ ),  $7\alpha$ , (25R)26-diHC ( $P = 0.05$ ) and  $7\alpha$ , (25R)26-diHCO

( $P = 0.01$ ). When plasma was analysed from these patients, none of the metabolites showed statistical differences compared to controls except  $7\alpha$ , (25R)26-diHCO, which unlike the situation in CSF was reduced in concentration in plasma ( $P < 0.05$ ). Little is known of the regulation of enzymes of the acidic pathway of bile acid biosynthesis [44]. However, the drive for an increased flux through the “acidic pathway” may be a consequence of increased availability of non-esterified cholesterol. In fact, the absence of a change in non-esterified cholesterol levels in CSF in all disease groups, except those with PBI or ALS, highlights its tight regulation in CNS. The increased CSF concentrations of metabolites of the “acidic pathway” of bile acid biosynthesis indicate that this pathway represents a salvage route for removal of excess cholesterol in CNS disease which may be defective or overstretched in ALS and PBI. The explanation for the increase in 25-HC in CSF of SA/UA patients may be an up-regulation of the IFN-stimulated gene *CH25H* in macrophages of the inflamed CNS.

### Pair-Wise Correlations between Sterols

It is of interest to study pair-wise correlations between CSF concentrations and specific sterol analytes which are arranged in metabolic order in Fig. 5. We have omitted the inflammatory CNS disease group from Fig. 5 as many correlations are driven by this group (see Online Resource 6 for  $P$  values for the significance of correlation (A) excluding and (B) including the inflammatory CNS disease group). As might be expected (25R)26-HC is highly correlated with  $7\alpha$ , (25R)26-diHC and  $3\beta$ -HCA corresponding to the first metabolites of the two branches of the “acidic pathway” of bile acid biosynthesis (Fig. 4). (25R)26-HC also correlates with  $7\alpha$ , (25R)26-diHCO the downstream metabolite of  $7\alpha$ , (25R)26-diHC and with  $3\beta,7\alpha$ -diHCA and  $3\beta,7\beta$ -diHCA, downstream metabolites of  $3\beta$ -HCA, but to a lesser extent. Concentrations of (25R)26-HC also correlate with 24S-HC, presumably as both have cholesterol as their precursor. Both 24S-HC and (25R)26-HC correlate strongly with cholesterol. 25-HC also correlates with (25R)26-HC but less strongly than 24S-HC.  $7\alpha$ , (25R)26-diHC correlates strongly with its downstream metabolite  $3\beta,7\alpha$ -diHCA, while  $7\alpha$ , (25R)26-diHCO correlates most strongly with  $7\alpha$ ,25-diHCO, both products of HSD3B7 metabolism.  $3\beta$ -HCA correlates very strongly with  $3\beta,7\beta$ -diHCA and less strongly with  $7\alpha$ H,3O-CA and  $3\beta,7\alpha$ -diHCA.  $3\beta,7\alpha$ -diHCA correlates most strongly with  $7\alpha$ , (25R)26-diHC and  $3\beta,7\beta$ -diHCA. This data suggest that the  $7\alpha$ , (25R)26-diHC branch provides the primary route to  $3\beta,7\alpha$ -diHCA in CNS.  $7\alpha$ H,3O-CA correlates with  $7\alpha$ H,3,24-diO-CA and the ultimate metabolite found in CSF,  $7\alpha$ -hydroxy-3-oxochole-4-enoic acid ( $7\alpha$ H,3O- $\Delta^4$ -BA). A fascinating feature of the pair-wise correlations is the tight cluster indicated by the “red box” incorporating  $7\alpha$ H,3O-CA,



$7\alpha\text{H},3\text{O-CA}$  correlates strongly with  $7\alpha\text{H},3\text{O-}\Delta^4\text{-BA}$ . As evident from the dendrogram in the Fig. in Online Resources 12, 25-HC clusters most strongly with 24S-HC and both cluster with their precursor cholesterol, while  $7\alpha,25\text{-diHC}$  and  $7\alpha,(25\text{R})26\text{-diHC}$  cluster together as do their HSD3B7 products  $7\alpha,25\text{-diHCO}$  and  $7\alpha,(25\text{R})26\text{-diHCO}$ .  $7\alpha,25\text{-diHC}$  and  $7\alpha,(25\text{R})26\text{-diHC}$  share CYP7B1 as the ultimate enzyme in their biosynthesis.

In summary, we have analysed plasma and CSF from patients with CIS and RRMS as well as from patients with inflammatory CNS disease, neurodegenerative disease and symptomatic control patients. In plasma we find that the concentration of 25-HC in RRMS patients is significantly lower than in controls. This is consistent with the recent report of Reboldi et al. that lower concentrations of 25-HC in *Ch25h*<sup>-/-</sup> mouse plasma results in reduced negative feedback by 25-HC on IL-1 family cytokine production and an exacerbated EAE, a rodent paradigm of MS [33]. In CSF, we find that the dominating cholesterol metabolites reflect the “acidic pathway” of bile acid biosynthesis and the elevated levels of these metabolites in CNS disease is likely to reflect cholesterol release as a result of demyelination or neuron death.

**Acknowledgements** This work was supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC, grant numbers BB/I001735/1 to WJG, BB/L001942/1 to YW). Members of the European Network for Oxysterol Research (ENOR, <http://oxysterols.com/>) are thanked for informative discussions.

**Compliance with Ethical Standards** Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and the study was approved by the Common Institutional Review Board of the Cantons of Basel, Switzerland.

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