Paper:
Griffiths, W., Abdel-Khalik, J., Yutuc, E., Roman, G., Warner, M., Gustafsson, J. & Wang, Y. (2019). Concentrations of bile acid precursors in cerebrospinal fluid of Alzheimer's disease patients. *Free Radical Biology and Medicine, 134*, 42-52.
http://dx.doi.org/10.1016/j.freeradbiomed.2018.12.020

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Concentrations of bile acid precursors in cerebrospinal fluid of Alzheimer's disease patients

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ARTICLE INFO
Keywords:
Sterol
Cholesterol
Oxysterol
Bile acid
Brain
Neurodegenerative disease
Mass spectrometry, Cytochrome P450

ABSTRACT
Using liquid chromatography – mass spectrometry in combination with derivatisation chemistry we profiled the oxysterol and cholestenoic acid content of cerebrospinal fluid from patients with Alzheimer's disease (n = 21), vascular dementia (n = 11), other neurodegenerative diseases (n = 15), Lewy bodies dementia, n = 3, Frontotemporal dementia, n = 11) and controls (n = 15). Thirty different sterols were quantified and the bile acid precursor 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid found to be reduced in abundance in cerebrospinal fluid of Alzheimer's disease patient-group. This was the only sterol found to be changed amongst the different groups.

1. Introduction
Cholesterol has been linked to the aetiology of Alzheimer's disease (AD) for decades with the rs4 allele of apolipoprotein E gene (APOE) being the most robust genetic risk factor for sporadic AD [1,2]. More recently cholesterol metabolism-related genes including ABCA7 (ATP binding cassette subfamily A member 7), ABG1 (ATP binding cassette subfamily G member 1), CLU (apolipoprotein J) and SORL1 (LDLR related with 11 ligand-binding repeats) have been classified among susceptibility loci by large genome-wide association studies (GWAS) [3,4]. Furthermore, in 2018 Picard et al. reported that polymorphism rs2269657 of the SREBF2 gene, which codes for the protein sterol regulatory element-binding protein-2 (SREBP-2), the master transcription factor regulating cholesterol biosynthesis, showed significant dual association with late-onset AD pathological biomarkers and gene expression levels [5]. Expression levels of the rs2269657 allele of SREBF2 in frontal cortex from late-onset AD brain inversely correlated with plaque density and with age at death [5].

Cholesterol is abundant in brain (2% wet weight) with about 25% of the total body cholesterol being found in brain [6]. Cholesterol cannot cross the blood brain barrier (BBB), hence after parturition essentially all cholesterol in brain is synthesised in situ from acetyl CoA, and all cholesterol export is via metabolism [6]. The first step of cholesterol metabolism is formation of an oxysterol, an oxidised form of cholesterol, and subsequent metabolism leads to steroid hormones and bile acids [7], i.e. in the central nervous system (CNS) to neurosteroids and C27 bile acids [8,9]. In brain the dominant oxysterol is 24S-hydroxycholesterol [10], formed in neurons by oxidation of cholesterol by CYP46A1 (cytochrome P450 family 46 subfamily A member 1) [11], this can be metabolised further in CNS [12] or exported as the intact molecule over the BBB [13]. Björkhem and colleagues have suggested the balance between 24S-hydroxycholesterol and its positional isomer (25R)26-hydroxycholesterol (common name 27-hydroxycholesterol) in brain may affect the production of beta-amyloid in brain [14]. (25R)26-Hydroxycholesterol may be formed via CYP27A1 (cytochrome P450 family 27 subfamily A member 1) mediated oxidation of cholesterol in brain or imported into brain from extracerebral sources [14-16]. (25R)26-Hydroxycholesterol is elevated in AD brain [17] and Björkhem et al. have suggested (25R)26-hydroxycholesterol may provide a missing link between hypercholesterolaemia and AD [14].

While it is difficult to investigate brain from living subjects, cerebrospinal fluid (CSF), the fluid which bathes the brain, is available. Papassotiropoulos et al. and Schönknecht et al. found elevated CSF concentrations of 24S-hydroxycholesterol in AD patients, which they explained by increased cholesterol turnover during neurodegeneration [18,19]. Surprisingly, this elevation is not evident in AD plasma [14].
CYP46A1 is normally expressed in neurons [11], but in AD is also expressed in glia cells [20]. 24S-Hydroxycholesterol reduces expression of enzymes of the cholesterol biosynthesis pathway in mouse neurons and glia, presumably by inhibiting the processing of SREBP-2 to its active form as a transcription factor, but up-regulates expression of APOE [21,22]. ApoE is a target gene of liver X receptors (LXRα and β, NR1H3 and NR1H2), both of which are expressed in mouse brain [23], and known to be activated by oxysterols, including 24S-hydroxycholesterol [24]. (25 R)26-Hydroxycholesterol has also been found to be elevated in CSF from AD patients [25,26].

The end products of cholesterol metabolism include bile acids and steroid hormones. Intermediates in the bile acid biosynthesis pathways have been observed in human and rodent CSF [8,9,12,27,28] and brain [29,30], while bile acids have been found in rodent [29,31,32] and human brain [33]. Pan et al. found levels of taurocholic acid were reduced in AD brain [33]. When plasma was analysed they found cholic acid to be reduced in AD patients [33]. Others have found lithocholic acid to be increased in plasma from AD patients [34], while in an un-targeted metabolomics study the glycine conjugates of cholic acid, deoxycholic acid and chenodeoxycholic acid have been found to be elevated in AD plasma [35].

In an effort to understand further the relationship between cholesterol metabolism and AD we have profiled the oxysterol content of CSF from AD patients, those with vascular dementia (VD) and other neurodegenerative disease (OND), and normal controls with no evidence of Alzheimer’s disease (Aβ) or possible NPH (these are considered “probable NPH”). A spinal tap was performed to confirm or not the diagnosis of NPH. After the spinal tap with removal of CSF (material analysed in the current study) the following diagnostic categories were concluded: (i) No evidence of NPH (these are considered “normal” controls, n = 15); cases of NPH plus (ii) AD, n = 21, (iii) OND, n = 15, including Lewy bodies dementia, LBD, n = 3; Frontotemporal Dementia, FTD, n = 11) and Vascular Dementia (VD, n = 11, Table S3). There was no statistically significant difference in concentration measured, as either ng/mL or ng/µg-cholesterol, in any of the oxysterols investigated between the different sample groups (Figs. 1 and 2, Supplemental Fig. S2).

2. Methods

2.1. Biomaterial

CSF samples were obtained from patients evaluated at the Memory Clinic, Methodist Hospital, Houston, because of complaints of memory loss. They were all found to have enlarged ventricles and were considered “probable NPH”. A spinal tap was performed to confirm or not the diagnosis of NPH. After the spinal tap with removal of CSF (material analysed in the current study) the following diagnostic categories were concluded: (i) No evidence of NPH (these are considered “normal” controls, n = 15); cases of NPH plus (ii) AD, n = 21, (iii) OND, n = 15, including Lewy bodies dementia, LBD, n = 3; Frontotemporal Dementia, FTD, n = 11) and Vascular Dementia (VD, n = 11, Table S3). There was no statistically significant difference in concentration measured, as either ng/mL or ng/µg-cholesterol, in any of the oxysterols investigated between the different sample groups (Figs. 1 and 2, Supplemental Fig. S2).

2.2. Methods

Sterols including oxysterols and bile acid precursors were analysed by liquid chromatography (LC) – mass spectrometry (MS) incorporating charge-tagging methodology, termed “Enzyme-Assisted Derivatization for Sterol Analysis” (EADSA) [9,36,37]. The method is fully described in Abdel-Khalik et al. [9]. In brief, sterols were extracted into ethanol from CSF, oxidised with cholesterol oxidase and derivatized with deuterated Girard P reagent ([2H₇]GP). A separate aliquot of extract was similarly derivatized with [2H₇]GP in the absence of enzyme (Supplemental Fig. S1). The derivatives were combined and analysed by LC-MS, exploiting high mass-resolution (120,000, full-width at half-maximum height definition) and multistage fragmentation (MS^n).

Quantification was achieved, where possible, by the addition of isotope-labelled standards. In the absence of an exact isotope-labelled standard quantification was made against isotope-labelled structural-analogues (See Supplemental Tables S2–S4) [9,37]. Crick et al. and Karu et al. have previously demonstrated the efficiency of the extraction, derivatization and purification methods and Crick et al. validated the method for multiple sterols and C₃₂ bile acids [37,38]. Here, despite the absence of an authentic isotope-labelled standard for 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid we have confirmed the validity of using [2H₇]24(R/S)-hydroxycholesterol as its internal standard by the standards addition method, adding consecutively increasing amounts of acid (0.2 ng/mL – 0.8 ng/mL) to a pooled CSF sample containing a set concentration of [2H₇]24(R/S)-hydroxycholesterol (8 ng/mL).

3. Results

In the current study we measured the concentrations of unesterified “free” sterols, oxysterols and bile acid precursors in the absence of saponification or solvolysis. This contrasts to most other studies of sterols and oxysterols by other groups where a solvolysis step is included to hydrolyse fatty acid esters [18,19,25]. Data for the control sample set has been reported previously in [9]. In the absence of authentic isotope-labelled standards for cholestanolic acids, [2H₇]24(R/S)-hydroxycholesterol was used as the internal standard. This selection has previously been validated by Crick et al. and here specifically for 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid using the standard additions method where a plot of measured concentration against theoretical concentration gave a straight line with R² > 0.994.

3.1. Oxyesters in CSF

The concentrations of monohydroxycholesterols, dihydroxycholesterols and dihydroxycholest-4-en-3-ones in CSF are low, ranging from about the limit of quantification of the method, 0.01 ng/mL, for the dihydroxycholestanes to about 0.1 ng/mL for (25 R)-26-hydroxycholesterol and 7β-hydroxycholesterol (Supplemental Tables S2 and S3). There was no statistically significant difference in concentration measured, as either ng/mL or ng/µg-cholesterol, in any of the oxysterols.

3.2. Bile acid precursors in CSF

As in other studies [8,9,12,27,28], we measured intermediates of the acidic pathway of bile acid biosynthesis in CSF (Supplemental Fig. S3). These include 3β-hydroxycholesterol-5-en-(25 R)-26-oic acid, its CYP7B1 (cytochrome P450 family 7 subfamily B member 1) metabolite, 3β,7α-dihydroxycholesterol-5-en-(25 R)-26-oic acid and the 3-oxo metabolite, 7α-hydroxy-3-oxocholest-4-en-(25 R)-26-oic acid, formed by oxidation at C-3 and Δ₅,Δ⁷ isomerisation by HSD3B7 (3β-hydroxy-cholesteroid dehydrogenase type 7). The latter compound is present at high levels (~28 ng/mL – 40 ng/µg-cholesterol) in CSF (Fig. 2, see also Supplemental Fig. S4 and Tables S2 & S3). 7α-Hydroxy-3-oxocholest-4-en-(25 R)-26-oic acid can be metabolised in the peroxisome to the CoA thioesters of 7α,24R-dihydroxy-3-oxocholest-4-en-(25 R)-26-oic acid, then 7α-hydroxy-3,24-bischolest-4-en-(25 R)-26-oic acid and
Fig. 1. Dot-plots displaying the concentrations of different oxysterols in CSF. Each dot indicates an individual patient sample. Concentrations are in ng/µg-cholesterol. The black bars indicate the mean value for each group. There were no statistical differences between the disease and control groups. (A) 24S-HC, 24S-hydroxycholesterol; (B) 25-HC, 25-hydroxycholesterol; (C) (25 R)26-HC, (25 R)26-hydroxycholesterol; (D) 25D₃, 25-hydroxyvitamin D₃; (E) 7α-HC, 7α-hydroxycholesterol; (F) 7β-HC, 7β-hydroxycholesterol; (G) 7O-C, 7-oxocholesterol. Abbreviations: - AD, Alzheimer's disease; VD, vascular dementia; OND, other neurodegenerative diseases (i.e. Lewy bodies dementia, Frontotemporal dementia).
Fig. 2. Dot-plots displaying the concentrations of different dihydroxysterols and cholestenoic acids in CSF. Each dot indicates an individual patient sample. Concentrations are in ng/µg-cholesterol. The black bar indicates the mean value. (A) 7α,(25R)26-diHCO, 7α,(25R)26-dihydroxycholest-4-en-3-one; (B) 7α,25-diHCO, 7α,25-dihydroxycholest-4-en-3-one; (C) 3β-HCA, 3β-hydroxycholest-5-en-(25 R)26-oic acid; (D) 3O-CA, 3-oxocholest-4-en-(25 R)26-oic acid; (E) 3β,7α-diHCA, 3β,7α-dihydroxycholest-5-en-(25 R)26-oic acid; (F) 7αH,3O-CA, 7α-hydroxy-3-oxocholest-4-en-(25 R)26-oic acid; (G) 3β,7β-diHCA, 3β,7β-dihydroxycholest-5-en-(25 R)26-oic acid.
ultimately 7α-hydroxy-3-oxochol-4-en-24-oic acid [30,39]. In our assay we observe the hydrolysed thioesters, but, in the absence of authentic standards of the different diasteriomers, could not determine the stereochemistry at C-25. 7α,24-Dihydroxy-3-oxocholest-4-en-26-oic acid could also be derived from 24S-hydroxycholesterol and have 24S-,25R- or 24S-,25S-stereochemistry [12].

A bile acid precursor prevalent in CSF is 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid (Fig. 3). The identity of this metabolite was confirmed by comparison of retention time, exact mass and MS3 spectra to that of the authentic standard 7α,25(R/S)-dihydroxy-3-oxocholest-4-en-26-oic acid. The concentration of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid falls in CSF from 2.04 ± 0.61 ng/mL in the control group to 1.63 ± 0.52 ng/mL in the AD group (Supplemental Fig. S5). This difference is statistically significant (P < 0.05). When measured in ng/µg-cholesterol the control group and AD group concentrations are 2.97 ± 1.12 and 2.09 ± 0.8, respectively, in which case P < 0.01 (Fig. 4). Of all the bile acid precursors this was the only one found to be changed significantly in any of the patient-groups.
3.3. Cholesterol

We also measured the levels of cholesterol and its precursors desmosterol and 7-dehydrocholesterol and the isomer 8-dehydrocholesterol. There was no statistical difference in concentrations of these sterols between the different sample groups (Fig. 5 and Supplemental Fig. S6 and Table S4).

3.4. Pairwise correlation between CSF levels of analytes

Spearman’s rank correlation can aid the determination of whether the levels of two analytes correlate, either positively or negatively. The higher or lower the statistical score for the correlation on a scale from +100 to −100, the more likely it is that the analytes correlate positively or negatively. Scores are called the correlation coefficients ($r$) [40,41]. Shown in Fig. 6 is a heat map of Spearman’s rank correlation coefficients ($r$) × 100 between the indicated pairs of analytes. A bipolar gradient between red (positive correlation) and blue (negative correlation) is indicated by the scale on the right-hand side of the Figure. Only values greater than ±40 show statistically significant correlation at a 1% significance level. Values lower than ±40 have been marked grey to show that these correlations are not statistically significant.

Perhaps not surprisingly, 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid positively correlates most strongly with other cholestenoic acids...
Fig. 5. Dot-plots displaying the concentrations of a cholestatrien-3β-ol, desmosterol and 8-dehydrocholesterol in CSF. Each dot indicates an individual patient sample. Concentrations are in ng/µg-cholesterol. The black bar indicates the mean value. (A) Triene, cholestatrien-3β-ol; (B) Des, desmosterol; (C) 8-DHC, 8-dehydrocholesterol.

Fig. 6. Heat map generated using the program R version 3.2.2 displaying pairwise correlations between cholesterol metabolite concentrations in human CSF samples. Values in the heat map are Spearman’s rank correlation coefficients \((r) \times 100\) between the indicated pairs of metabolites. A bipolar gradient between red (positive correlation) and blue (negative correlation) is used. Only values greater or lesser than \(\pm 40\) show statistically significant correlation at a 1% significance level. Values within \(\pm 40\) have been marked grey to show that these correlations are not statistically significant. Abbreviations for cholesterol metabolites are as in Figure Captions 1 – 5 and Supplemental Table S2.
and the C24 acid 7α-hydroxy-3-oxochol-4-en-24-oic acid. Interestingly, it also correlates positively with 7α,(25 R)26-dihydroxycholest-4-en-3-one, 7α,25-dihydroxycholest-4-en-3-one and (25 R)26-hydroxycholesterol.

4. Discussion

A limitation of the present study is a lack of isotope-labelled authentic standards for many of the metabolites studied, necessitating the use of structural analogues (See Supplemental Tables S2–S4). Earlier studies by Crick et al. have indicated that this is a valid approach when incorporating GP-derivatisation which equalises for structure-specific variation in ionisation efficiency by incorporating a permanent positive-charge in the analyte [37]. Crick et al. have also shown that in the absence of isotope-labelled standards correction for analyte loss during sample preparation can be made by the use of standards with similar hydrophobicity [37]. Here we have confirmed this for 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid by performing a standard additions experiment, where R² for the plot of measured concentration against theoretical concentration was found to be > 0.994.

We have previously reported the presence of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid in human CSF [9,12], where, as here, the identification was made based on retention time, exact mass and MS³ spectra of the GP-derivative and comparison to the authentic standard which was available as a mixture of 25R- and 25S-epimers. In the present study, and those made earlier, we cannot be sure of the exact stereochemistry at C-25 of the acid found in CSF. The origin of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid is possibly via CYP3A4 (cytochrome P450 family 3 subfamily A member 4) catalysed 25-hydroxylation after 7α-hydroxylation and before, or after, C-26-hydroxylation and -carboxylation by CYP27A1 (Fig. 7, see also Supplemental Fig. S7) [12]. This would be consistent with 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid correlating positively with 7α,25-dihydroxycholest-4-en-3-one. We have demonstrated that CYP3A4 hydroxylates 7α-hydroxycholesterol [42], while Honda et al. have shown that CYP3A4, like CH25H (cholesterol 25-hydroxylase), is a 25-hydroxylase [43]. Alternatively, the start of the biosynthesis pathway for 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid may be 25-hydroxylation of cholesterol by CH25H [44].

The correlation data presented in Fig. 6 is consistent with 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid being formed via the enzymes CYP3A4, CYP27A1, HSD3B7 and either CYP7A1 or CYP7B1 (Fig. 7, Supplemental Fig. S7). With the exception of CYP7A1, each of these enzymes is known to be expressed in human brain [8,45–49], while 7α-hydroxycholesterol is known to be present in rodent brain, presumably originating in the periphery and crossing the BBB into brain or being derived from non-enzymatic oxidation of cholesterol in brain [42]. CYP27A1 the enzyme required to introduce the carboxylic acid group at C27 is expressed in neurons, oligodendrocytes and some astrocytes in human brain, and in AD brain its expression is reduced in neurons but increased in oligodendrocytes, perhaps reflecting a decrease and increase in their respective cell numbers [46]. CYP3A4 is expressed in neurons, primarily localized in the soma and axonal hillock [45] and CYP7B1 is also expressed in neurons [49]. Yau et al. have shown that in AD brain the per neuron levels of CYP7B1 mRNA are reduced in hippocampal sections, suggesting a selective impairment in ability of AD brain to 7α-hydroxylate oxysterols [49]. Conversely, HSD3B7 is not expressed in neurons, only glia [48]. From the above we suggest that
the reduced level of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid in AD CSF is a consequence of a reduced synthesis, resulting from a loss of 3β,7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid or be reduced at Δ4 by AKR1D1 (aldo-keto reductase family 1 member D1) then at C-3 by AKR1C4 (aldo-keto reductase family 1 member C4) prior to C-24 carbonylation and elimination of 2-oxo-propanoic acid (Fig. 7). Although AKR1D1 and I4C are usually regarded as liver specific, Mano et al. have shown that enzymes in the cytosolic fraction prepared from rat brain can convert 7α-hydroxy-3-oxocholest-4-en-24-oic acid to chenodeoxycholic acid [55]. Whether these enzymes are present in human brain and can similarly act on C₂₅ acids is unknown. Further studies of CSF targeting additional metabolites are required to learn more about the metabolism of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid.

The authentic standard of 3β,7α,25-trihydroxycholesterol-5-en-26-oic acid, from which 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid is synthetically derived by bacterial cholesterol oxidase treatment, was only recently custom synthesised by Avanti Polar Lipids Inc. We await synthesis of an isolate-labelled version to fully validate the current findings on a larger cohort of AD patients.

Previous studies have shown that the concentration of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid in CSF is higher than in serum [9], and we suggest that in the healthy brain 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid provides a route for removal of cholesterol via metabolism to more a hydrophilic metabolite. Its reduced concentration in the CSF of the AD patient-group with AD. Based on LC-MS identifications of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid directly contributes to the pathophysiology of AD, as very little is known of the biological activities of this molecule.

How 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid is metabolised is not known. It may fall into the Duane bile acid biosynthesis pathway [54] with formation of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid and elimination of 2-oxo-propanoic acid with formation of 7α-hydroxy-3-oxocholest-4-en-24-oic acid and ultimately chenodeoxycholic acid or be reduced at Δ4 with formation of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid provides a route for removal of cholesterol via metabolism to more a hydrophilic metabolite. Its reduced concentration in the CSF of the AD patient-group suggests that this route is attenuated in this disease state. It cannot be ruled out that, like many other cholesterol-derived molecules, 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid may also have signalling properties which become diminished in the disease state. In light of the reduced level of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid in CSF of the AD patient-group it is tempting to devise routes to enhance its formation. Although perhaps a naïve suggestion, if CYP3A4 is the necessary 25-hydroxylase this could be up-regulated through activation of the pregnane X receptor (PXR, NR1I2) or the constitutive androstane receptor (CAR, NR1I3), as CYP3A4 is a target gene of both PXR and CAR [56,57]. PXR is activated by multiple agonists including dexamethasone, rifampicin and enzyme-inducing anti-epileptics, as well as many constituents of herbal remedies, while CAR is activated by several environmental chemicals and pharmaceuticals [58–60]. In fact, the effects in human of PXR activation by rifampicin, also known as rifampin, which demonstrates good CNS penetration [61], have recently been assessed in a randomized, open, placebo-controlled crossover trial on an oral glucose tolerance test [62]. The PXR agonists elicited postprandial hyperglycaemia, suggesting a detrimental role of PXR activation on glucose tolerance. Although none of the participants reached the clinical criterion of impaired glucose tolerance [62], there are obvious limitations of this approach. Alternatively, cholesterol 25-hydroxylase, CH25H, an interferon-stimulated gene, may be activated via the interferon receptor [63]. Interestingly, Merck KGaA have performed a clinical trial (ClinicalTrials.gov Identifier: NCT01057563) to evaluate interferon β-1a in the treatment of AD.

In summary, we have identified a statistically significant reduction of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid in the CSF of the patient-group with AD. Based on LC-MS identification of cholesterol metabolites and correlations of their concentrations, we suggest mechanisms for the formation of 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acid. Interestingly, one of the suggested pathways relies on CH25H, an interferon stimulated gene implicated in AD.

Acknowledgements

This work was supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC, grant numbers BB/1001735/1 and BB/N015932/1 to WJG, BB/L001942/1 to YW), an Engineering and Physical Sciences Research Council Impact Acceleration Account (to Swansea University), the Welsh Government (A4B grant to WJG and YW), the Swedish Science Council (grant to JÅG) and the Robert A. Welch Fund (Grant E-0004 to JÅG). JAK was supported by a Ph.D. studentship from Imperial College Healthcare Charities. Members of the European Network for Oxysterol Research (ENOR, https://www.oxysterols.net/) are thanked for informative discussions.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.12.020.

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51

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51

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