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RESEARCH

Sleep quality and cerebrospinal fluid oxysterols in degenerative dementias: correlations and possible biomedical implications

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Abstract

Objective: The combination and tentative correlation of a selected set of polysomnographic tests with the quantitative measurement of oxysterols of pathophysiological relevance in the cerebrospinal fluid (CSF) of patients with Alzheimer's degenerative dementia (AD), non-Alzheimer's degenerative dementia (NAD) and nondegenerative disorders (C) were afforded in a pilot study.

Methods: Sleep efficiency, percentage of sleep time spent in nonrapid eye movement (NREM) stage 3 (N3), apnea/hypopnea index and sleep time spent with oxygen saturation <90% were recorded. Oxysterols of both enzymatic and nonenzymatic origin were quantified in the CSF of the three groups of patients by isotope-dilution gas chromatography mass spectrometry.

Results: A remarkable increase in all tested oxysterols of autoxidation origin and of cholesterol was detectable in the CSF of AD and NAD patients in comparison with C patients. Of the four markers of sleep quality tested, only the percentage duration of N3 showed a net progressive reduction in NAD and AD patients. A strong inverse correlation between the CSF levels of 7k-cholesterol (7KC) and 7α-hydroxycholesterol and the duration of N3% in the recruited cohorts of patients appeared evident.

Conclusions: An abnormal increase in some oxysterols of autoxidation origin, particularly of 7KC, in the CSF of patients with degenerative dementia, with the highest levels reached in AD patients, appears to be a reliable candidate biomarker of reduced duration of N3 sleep. The observed accumulation of oxysterols in the CSF seems at least in part because of impaired efficiency of the glymphatic system, which indeed reaches, under normal conditions, its maximum activity in the N3 sleep phase. A contribution of altered cholesterol metabolism to sleep quality in AD patients cannot be excluded.

Significance statement

Oxysterols of nonenzymatic origin are proposed as new CSF candidate markers of sleep quality in patients with dementia.

Keywords: Alzheimers' disease; degenerative dementia; sleep disorders; cerebrospinal fluid; oxysterols; NREM stage 3 (N3)

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Introduction

Sleep disorders are common and of early occurrence in individuals with suspected Alzheimer's disease (AD) (Moran *et al.* 2005). Their actual contribution to the pathogenesis and progression of this neurodegenerative disease has been so far debated and poorly elucidated. Changes to the sleep start often before the onset of cognitive impairment (preclinical AD), as evidenced by a recent meta-analysis (Shi *et al.* 2018). However, at the symptomatic stage, the quality of sleep worsens constantly, suggesting that sleep impairment and the neurodegenerative process are concomitant and may be related to each other (Ju *et al.* 2014). Thus, in our investigation, we combined polysomnography (PSG) and clinical pathology.

Another meta-analysis study on the polysomnographic findings achieved in AD patients compared with controls once more provided solid evidence of the occurrence of several sleep disturbances in AD, including a significant reduction in total sleep time, sleep efficiency and percentage of slow-wave sleep or nonrapid eye movement (NREM) stage N3 (slow-wave sleep or N3) (Zhang *et al.* 2022). Of note, there was an evident association of N3 reduction with the severity of cognitive impairment in AD patients (Zhang *et al.* 2022). Moreover, sleep disorders are also associated with non-Alzheimer's degenerative dementias (NADs) such as Parkinson's disease (PD), Huntington's disease and frontotemporal dementia (Malhotra 2018), and precocious N3 instability has been demonstrated at least in PD, even at early stages of the disease (Priano *et al.* 2019).

The glymphatic system, i.e. the paravascular pathway that allows the cerebrospinal fluid (CSF) to flow in the brain parenchyma, removing waste products and interstitial solutes including amyloid β (Iliff *et al.* 2012, Jessen *et al.* 2015, Hauglund *et al.* 2020), shows its maximum activity during N3 (Hablitz & Nedergaard 2021, Voumvourakis *et al.* 2023).

It has been clearly demonstrated in humans that experimental sleep disruption consistently triggered the key signaling pathway of inflammation such as those involving nuclear factor-κ-B and activator protein-1 (Irwin & Vitiello 2019). Thus, the impairment of the physiological activity of the glymphatic system consequent to a reduction of N3 as observed in AD and PD could significantly contribute to the accumulation of pro-inflammatory molecules in the brain parenchyma, most likely not limited to amyloid β, of which the neuroinflammatory properties have long been proven (Glass *et al.* 2010, Hampel *et al.* 2021).

Several cholesterol oxidation products with both an enzymatic and a nonenzymatic origin, termed oxysterols, provided with neuroinflammatory properties, accumulate with AD progression (Testa *et al.* 2014, 2016). A progressive accumulation of 27-hydroxycholesterol (27OHC), an oxysterol of enzymatic origin, which passes from circulation into the brain (Leoni *et al.* 2005), was observed in postmortem AD brains, together with 7-ketocholesterol (7KC), 7α-hydroxycholesterol (7αOHC), 7β-hydroxycholesterol (7βOHC), 5α,6α-epoxycholesterol (α-epoxyC) and 5β,6β-epoxycholesterol (β-epoxyC). The AD brain content of 24-hydroxycholesterol (24OHC), another oxysterol of enzymatic origin, showed an opposite trend, i.e., a decrease directly related to disease progression (Testa *et al.* 2016). Mostly released by damaged and dead brain cells, these compounds are detectable in the CSF as well (Leoni & Caccia 2011) even if the quantification of oxysterols in this biological fluid has so far been essentially limited to the two side-chain oxysterols 27OHC and 24OHC, both shown for many years increased in the CSF of AD patients (Björkhem *et al.* 2006, Leoni & Caccia 2011).

The aims of this pilot study were as follows: i) the quantification of some oxysterols of pathophysiological interest in the CSF of patients with AD and the comparison of the results achieved with identical measurements in the CSF of patients with NAD and patients with nondegenerative disorders (C), the latter taken as reference control; ii) the combination of the clinical pathology data obtained with PSG analyses in the three groups of recruited patients and finally iii) the correlation between the amount of the individual oxysterols detected in the CSF and the different sleep biomarkers measured.

Materials and methods

Patients

Forty-one participants diagnosed with AD, NAD and C were enrolled in this pilot study. Each patient underwent the usual clinical workup that includes a multidisciplinary assessment with a neurological and neuropsychological evaluation, magnetic resonance imaging scan, routine laboratory assessment and a lumbar puncture to determine CSF biomarkers as part of the diagnostic procedure. The diagnosis of dementia was made using the standard diagnostic criteria (Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V)) and the NIA-AA criteria (American Psychiatric Association 2013, McKhann *et al.* 2011). The recruited patients were divided into 3 subgroups: 11 patients with AD, 22 patients with NAD and 8 patients with non-neurodegenerative disorders taken as the internal control (C). In Supplementary Table 1S (see section on Supplementary materials given at the end of the article), the diagnosis of the individual patients enrolled in the pilot study is reported.

CSF collection and storage

All 41 patients underwent lumbar puncture according to standard procedures. All spinal taps were performed in the morning, between 9 and 10 AM. The CSF sample

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was centrifuged at room temperature for 10 min at 2,000 *g*, aliquoted and stored at −80 °C until analysis, according to international biomarker recommendations (Vanderstichele *et al.* 2012).

Analyses of CSF AD biomarkers

The CSF total tau (t-Tau), phosphorylated tau (p-Tau_{181}) and amyloid beta 1–42 (Aβ42) levels were measured separately, in duplicate, using commercially available sandwich enzyme-linked immunosorbent assay kits (Innotest, Innogenetics/Fujirebio, Belgium). Intra-assay and interassay coefficients of variation were 3.2 and 11.5% for t-Tau, 1.7 and 11.4% for p-Tau_{181} and 4.6 and 7.8% for Aβ42. All the assays were performed according to the manufacturer's protocols. For the interpretation of the CSF biomarker results, the following cutoff values were considered: t-Tau > 300 ng/L for subjects aged 21–50 years, t-Tau > 450 ng/L for subjects aged 51–70 years and t-Tau > 500 ng/L for subjects over 71 years old; Aβ42 > 500 pg/mL according to Sjogren and coworkers (Sjögren *et al.* 2001); and p-Tau_{181} < 61 pg/mL according to Vanderstichele and coworkers (Vanderstichele *et al.* 2006). Consistent with the diagnostic criteria for AD, a CSF biomarker profile was considered suggestive for AD if the CSF Aβ1–42 value was below the cutoff, in combination with t-Tau and/or p-Tau_{181} values being above the threshold (Dubois *et al.* 2014). In some cases, the p-Tau/Aβ1–42 ratio was used for the determination of AD (cutoff value 0.08) (Duits *et al.* 2014).

CSF quantification of cholesterol, cholesterol precursors and oxysterols

Sterols and oxysterols were quantified by isotope-dilution gas chromatography mass spectrometry (Leoni *et al.* 2005, Marcello *et al.* 2020). To a screw capped vial sealed with a Teflon septum, 500 μL CSF were added together with structural homologous internal standards: 10 μg epicoprostanol (Sigma-Aldrich, Canada), 250 ng lathosterol-d7 (d7-latho), 20 ng lanosterol-d6 (d6-lamo), 20 ng 7αOHC-d7 (d7-7αOHC), 20 ng 7βOHC-d7 (d7-7βOHC), 20 ng 7KC-d7 (d7-7KC), 20 ng α-epoxyC-d7 (d7-α-epoxyC), 20 ng 5β,6β-epoxycholesterol-d7 (d7-β-epoxyC), 20 ng cholestane-3β,5α,6β-triol-d7 (d7-triol), 20 ng 24(*R*/*S*)-hydroxycholesterol-d7(d7-24OHC), 20 ng 27OHC-d6 (d6-27OHC) (Avanti Polar Lipids Inc., USA), 50 μL butylated hydroxytoluene (5 g/L, Sigma-Aldrich, Canada) and 50 μL K3-EDTA (10 g/L, Sigma-Merck) to prevent auto-oxidation; ethanol; and KOH 1 M. Each vial was flushed with argon for 10 min to remove air. Hydrolysis was carried out at room temperature, and then, sterols and oxysterols were extracted twice with 5 mL hexane. The two phases were evaporated under nitrogen stream and derivatized with BSTFA + 1% TCS at 70 °C and injected into GCMS.

Analyses were performed by isotope-dilution gas chromatography mass spectrometry with a DB-XLB column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific Alltech, USA) in an HP 6890N Network GC system (Agilent Technologies, USA) connected with a direct capillary inlet system to a quadrupole mass selective detector HP5975B inert MSD (Agilent Technologies, USA). The GC system was equipped with an HP 7687 series autosampler and an HP 7683 series injector (Agilent Technologies, USA).

For sterols and oxysterols, the oven temperature program was as follows: initial temperature of 180 °C was held for 1 min, followed by a linear ramp of 20 °C/min to 270 °C and then a linear ramp of 5 °C/min to 290 °C, which was held for 11 min. Helium was used as the carrier gas at a flow rate of 1 mL/min, and 1 μL sample was injected in the splitless mode. Injection was carried out at 250 °C with a flow rate of 20 mL/min. Transfer line temperature was 290 °C. Filament temperature was set at 150 °C and quadrupole temperature at 220 °C according to the manufacturer's indication.

Mass spectrometric data were acquired in the selected ion monitoring mode at m/z = 370 for epicoprostanol, m/z = 368 for cholesterol, m/z = 465 for d7-latho, m/z = 458 for lathosterol, m/z = 343 for desmosterol, m/z = 399 for d6-lamo, m/z = 393 for lanosterol, m/z = 463 for d7-7αOHC, m/z = 456 for 7α-OHC, m/z = 463 for d7-7βOHC, m/z = 456 for 7βOHC, m/z = 479 for d7-7KC, m/z = 472 for 7KC, m/z = 481 for d7-α-epoxyC, m/z = 474 for α-epoxyC, m/z = 481 for d7-β-epoxyC, m/z = 474 for β-epoxyC, m/z = 410 for d7-triol, m/z = 403 for triol, m/z = 413 for d7-24OHC, m/z = 413 for 24OHC, m/z = 462 for d6-27OHC and m/z = 456 for 27OHC.

Peak integration was performed manually, and oxysterols were quantified from selected-ion monitoring analysis against internal standards using standard curves for the listed sterols.

CSF quantification of malondialdehyde

The malondialdehyde (MDA) content in the CSF samples was determined by high-performance liquid chromatography using kits from Chromsystems (Chromsystems Instruments & Chemicals, GmbH, Germany). The inter- and intra-assay coefficients of variation were 5.4–8.8% and 0.9–2.9% respectively.

Sleep investigations and PSG

All recruited patients underwent an extensive sleep investigation that included clinical assessment and attended PSG according to the standard procedure. All patients underwent an adaptation night and then full-night PSG in the sleep laboratory in a quiet room under video monitoring. Patients were allowed to maintain their regular sleep habits and timing. The following parameters were recorded: EEG using C3–A2, C4–A1, O2–A1 and O1–A2 derivations integrated by bipolar montages Fp2–F4, F4–C4, C4–P4 and P4–02; Fp1–F3, F3–C3, C3–P3 and P3–01; and

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Table 1 Mean values ± SD of the clinical pathology and polysomnographic parameters measured in the three groups of patients: AD, NAD and C.

				P values		
Parameters	**AD**	**NAD**	**C**	**AD vs C**	**NAD vs C**	**AD vs NAD**
Patients (*n*)	11	22	8			
t-Tau (pg/mL)	446.0 ± 233.0	361.1 ± 188.7	182.7 ± 131.4	<0.05	NS	NS
p-Tau_{181} (pg/mL)	69.1 ± 31.7	65.3 ± 47.6	30.0 ± 21,8	NS	NS	NS
$A\beta_{42}$ (pg/mL)	484.8 ± 80.8	908.4 ± 290.3	952.9 ± 297.4	<0.001	NS	<0.001
MDA (μg/L)	8.5± 1.0	7.8 ± 1.7	7.6 ± 1.9	NS	NS	NS
Lathosterol (ng/mL)	1846.8 ± 423.4	1649.5 ± 342.9	1143.2 ± 324.7	<0.001	<0.01	NS
Desmosterol (ng/mL)	1234.0 ± 192.9	978.2 ± 205.4	699.6 ± 107.4	<0.001	<0.01	<0.01
Lanosterol (ng/mL)	178.6 ± 45.7	148.0 ± 62.0	73.7 ± 17.9	<0.001	<0.01	NS
Cholesterol (mg/L)	5676 ± 1024	4063 ± 960	2880 ± 661	<0.001	<0.01	<0.001
7αOHC ((ng/mL)	3.7 ± 0.6	2.6 ± 0.6	1.5 ± 0.3	<0.001	<0.001	<0.001
7βOHC (ng/mL)	4.0 ± 0.8	2.5 ± 0.6	1.6 ± 0.3	<0.001	<0.01	<0.001
7KC (ng/mL)	7.4 ± 1.0	5.2 ± 1.6	2.4 ± 0.4	<0.001	<0.001	<0.001
α-EpoxyC (ng/mL)	1.6 ± 0.4	1.2 ± 0.3	0.6 ± 0.1	<0.001	<0.001	<0.001
β-EpoxyC (ng/mL)	1.2 ± 0.3	1.0 ± 0.2	0.5 ± 0.1	<0.001	<0.001	NS
Triol (ng/mL)	1.4 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	<0.001	<0.01	<0.001
24OHC (ng/mL)	3.4 ± 0.3	3.1 ± 0.7	2.2 ± 0.2	<0.001	<0.001	NS
27OHC (ng/mL)	1.3 ± 0.3	1.3 ± 0.3	0.9 ± 0.1	<0.01	<0.01	NS
Sleep efficiency (%)	63.3 ± 7.2	64.7 ± 16.7	76.7 ± 12.2	NS	NS	NS
N3 (%)	12.9 ± 4.4	16.2 ± 4.3	23.8 ± 5.5	<0.001	<0.01	NS
AHI (events/h)	22.7 ±21.5	28.0 ± 21.6	21.6 ± 30.8	NS	NS	NS
TST90 (%)	34.0 ± 38.4	16.4 ± 17.8	10.3 ± 18.4	NS	NS	NS

Abbreviations: 7αOHC, 7α-hydroxycholesterol; 7βOHC, 7β-hydroxycholesterol; 7KC, 7-ketocholesterol; α-epoxyC, α-epoxycholesterol; β-epoxyC, β-epoxycholesterol; 24OHC, 24-hydroxycholesterol; 27OHC, 27-hydroxycholesterol; $A\beta_{42}$, amyloid β_{42}; AD, Alzheimer's dementia; AHI, apnea/hypopnea index; C, nondegenerative disorders; N3, NREM stage 3; NAD, non-Alzheimer's degenerative dementia; NREM, nonrapid eye movement; p-Tau_{181}, phosphorylated tau; t-Tau, total tau protein and TST90, total sleep time spent with oxygen saturation <90%.

Fz–Cz and Cz–Pz of the 10–20 international placement system; electrooculogram (bipolar montage: right ocular canthus–left ocular canthus); electrocardiogram; respiratory effort using thoracic and abdominal strain gauges, nasal air-flow using a nasal cannula, snoring using a microphone and arterial oxyhemoglobin (SaO_2) using a pulse oximeter with finger probe; and submental and tibialis anterior muscle electromyogram.

Conventional sleep analysis, respiratory parameters and arousals were performed independently by two evaluators experienced in sleep staging according to the literature (Iber *et al.* 2007). Among all the polysomnographic parameters, consistent with sleep alterations reported in AD or neurodegenerative disorders and the aim of the present study, we chose to focus the analysis in particular on a subset of sleep variables: sleep efficiency (SE), calculated as total sleep time/time in bed in percentage; percentage of total sleep time spent in N3; apnea/hypopnea index (AHI), defined as the number of apneas plus hypopneas/hours of sleep; and percentage of total sleep time spent with oxygen saturation <90% (TST90).

Statistical analysis

Statistical analyses were performed using Jamovi software (Version 2.3.28; https://www.jamovi.org/download.html). Data were tested for normality using Shapiro–Wilk's test. Differences between groups were analyzed using one-way ANOVA with Tukey's *post hoc* test. Correlations among variables were performed using Pearson's correlation test. The significance level was set to 0.05.

Results

The results of the biochemical and molecular biology analyses performed on the CSF of the 41 participants in the pilot study are summarized in Table 1 expressed as mean values ± SD, with relative *P* values. The CSF values of Aβ1–42, t-Tau and p-Tau_{181} measured in the three groups are congruent with the diagnosis of AD, NAD and C respectively. Unlike t-Tau, which is considered a marker of neuronal damage, Aβ1–42 is considered a specific biomarker of Alzheimer's disease and in the present study, Aβ1–42 well differentiates the AD group from the NAD one, reflecting the different composition (Sjögren *et al.* 2001).

Differential increase in oxysterols of nonenzymatic origin in the CSF of patients with AD and NAD

In this study, we compared the quantification of the main oxysterols previously detected in autoptic brain samples

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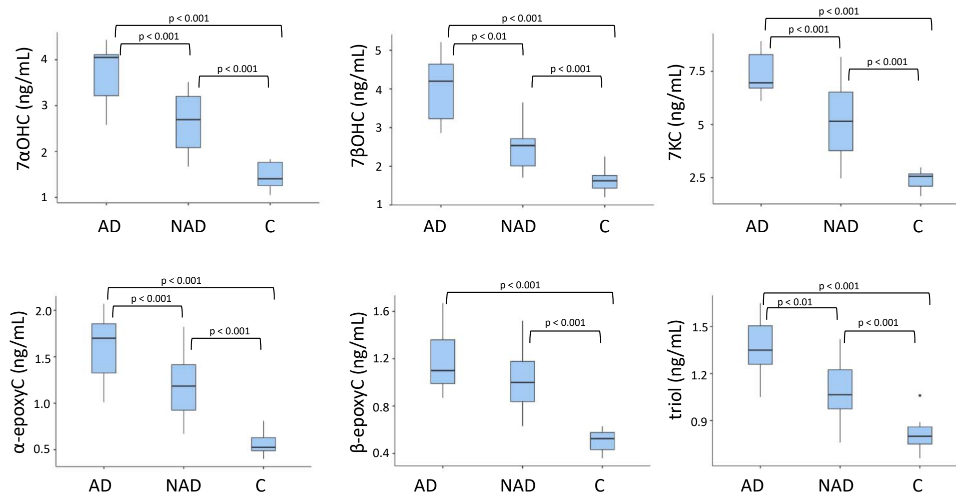

Figure 1

Oxysterols of nonenzymatic origin quantified in the cerebrospinal fluid (CSF) of patients with diagnosed Alzheimer's dementia (AD), non-Alzheimer's degenerative dementia (NAD) and nondegenerative disorders (C). After suitable CSF collection and storage, the quantitative analysis of the main oxysterols of nonenzymatic origin was performed by isotope-dilution gas chromatography mass spectrometry (see Materials and methods section). Box plots visually show the distribution of the data collected, calculated as means ± SD (see Table 1 for relative numerical details). 7αOHC, 7α-hydroxycholesterol; 7βOHC, 7β-hydroxycholesterol; 7KC, 7-ketocholesterol; α-epoxyC, α-epoxycholesterol; β-epoxyC, β-epoxycholesterol; triol, cholestane-3β,5α,6β-triol. An outlier value in the triol measurement was plotted as a dot. Significant *P* values were calculated by comparing the three groups of patients, AD, NAD and C. Only significant *P* values (<0.05) were shown in the diagrams.

from patients with AD or without neurodegenerative diseases (Testa *et al.* 2016) in the CSF of patients with diagnosed AD and NAD with those achieved in the CSF of individuals with nondegenerative diseases, considered as the control group (C).

As shown in Fig. 1, we observed elevated concentrations of several oxysterols in the CSF collected from both patients with diagnosed AD and NAD compared with the control group: the oxysterols of nonenzymatic origin 7α-OHC, 7β-OHC, 7KC, α-epoxyC, β-epoxyC and triol showed a CSF level from 1.5 to 3.0 times higher than that recovered in control CSFs. The two oxysterols of enzymatic origin 24OHC and 27OHC were significantly increased as well, 1.5 and 1.4 times, respectively, in AD and NAD compared with the C group (Fig. 2).

Furthermore, the CSF concentrations of 7α-OHC, 7β-OHC, 7KC, α-epoxyC and triol were significantly higher in CSF collected from AD patients compared with those of NAD patients (Fig. 1). On the other hand, the CSF levels of both enzymatic oxysterols measured in AD and NAD patients, i.e. 24OHC and 27OHC, did not significantly differ from each other (Fig. 2).

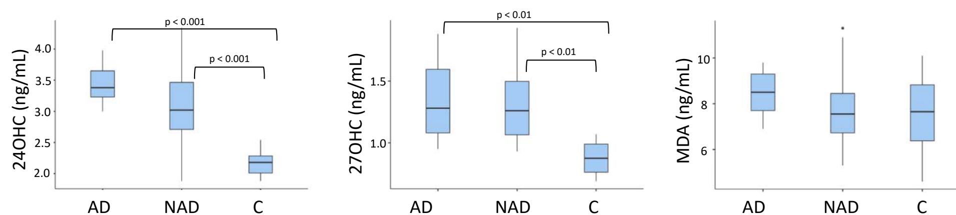

Figure 2

Oxysterols of enzymatic origin and malondialdehyde quantified in the cerebrospinal fluid (CSF) of patients with diagnosed Alzheimer's dementia (AD), non-Alzheimer's degenerative dementia (NAD) and nondegenerative disorders (C). After suitable CSF collection and storage, the quantitative analysis of 24-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC) was performed by isotope-dilution gas chromatography mass spectrometry (see Materials and methods). The determination of malondialdehyde (MDA) in the CSF samples was made by high-performance liquid chromatography (HPLC) (see Materials and methods). Box plots visually show the distribution of the data collected, calculated as means ± SD (see Table 1 for relative numerical details). An outlier value in the MDA measurement was plotted as a dot. Significant *P* values were calculated by comparing the three groups of patients, AD, NAD and C. Only significant *P* values (<0.05) were shown.

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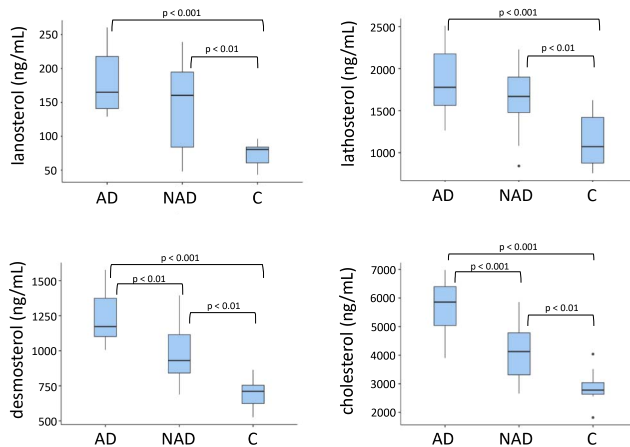

Figure 3

CSF content of cholesterol and its precursors lanosterol, lathosterol and desmosterol in AD, NAD and C groups of patients. After suitable CSF collection and storage, the quantitative analysis of the content of the three steroid precursors of cholesterol and of cholesterol as well was performed by isotope-dilution gas chromatography mass spectrometry (see Materials and methods). Box plots visually show the distribution of the data collected, calculated as means ± SD (see Table 1 for relative numerical details). Outliers were plotted as dots in lathosterol and cholesterol graphs. Significant P values were calculated by comparing the three groups of patients, AD, NAD and C. Only significant P values (<0.05) were shown.

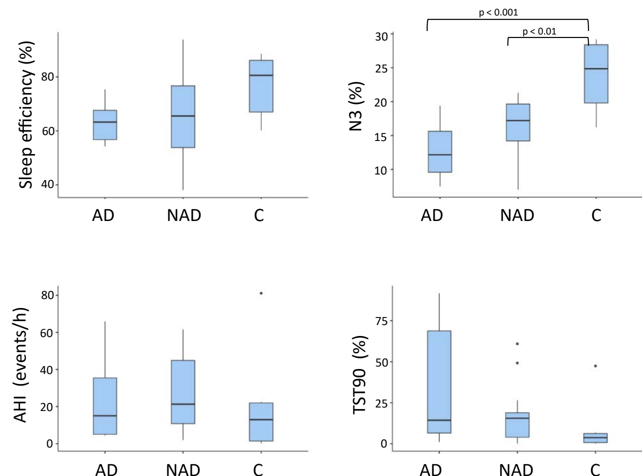

Figure 4

Performance of a selected subset of polysomnographic parameters in AD, NAD and C groups of patients. The selected markers of sleep disorders were sleep efficiency, calculated as percentage of total sleep time/time in bed; percentage of total sleep time spent in NREM stage 3 (N3%); apnea/hypopnea index (AHI); and percentage of total sleep time spent with oxygen saturation <90% (TST90). Box plots visually show the distribution of the data collected, calculated as means ± SD (see Table 1 for relative numerical details). Outliers were plotted as dots in AHI and TST90 diagrams. Significant P values were calculated by comparing the three groups of patients, AD, NAD and C. Only significant P values (<0.05) were shown.

The old classical index of oxidative stress MDA was measured in the CSF of the three groups of patients: no significant differences were observed between C and patients with dementia and between AD and NAD (Fig. 2).

Altered CSF levels of cholesterol and main steroid precursors in AD and NAD patients versus the internal control group

To complete the oxysterol measurement, cholesterol and its precursors lanosterol, lathosterol and desmosterol, markers of the cholesterol biosynthesis, were also quantified. As shown in Fig. 3, all four sterols were significantly increased in the CSF of AD and NAD patients compared with the CSF of the patients without any neurodegenerative disease, considered as the internal control. Moreover, the cholesterol content in the CSF of AD patients appeared significantly higher than that observed in NAD ones, mainly because of the increase in desmosterol (Fig. 3).

Different trends of the deep sleep phase N3 biomarker in AD and NAD patients versus the internal control group

In our study, we selected SE, the percentage of total sleep time spent in N3, AHI and the percentage of TST90 as the sleep markers.

As depicted in Fig. 4, only the N3 sleep biomarker showed a statistically significant difference between patients with dementia and healthy controls, both in the AD group ($P < 0.001$) and in NAD group ($P < 0.01$). A further reduction of N3 was observed in AD compared with NAD patients, but a statistically significant difference was not achieved, most likely because of the limited size of the patient groups and the variability observed in this pilot study (Fig. 4).

Strong inverse correlation between the CSF levels of 7KC and 7αOHC and the duration of N3 in the recruited cohorts of patients

Correlation analyses considering all the patients together, between the CSF oxysterols and the four sleep biomarkers, showed significant results essentially regarding the large majority of CSF oxysterols and percentage of N3.

Indeed, a strongly significant inverse correlation was observed between N3 duration and the CSF level of 7KC ($r = -0.7183$) and 7αOHC ($r = -0.6716$) (Fig. 5). Moderate inverse correlations (ranging between $r = 0.5$ and 0.6) were found for the other tested oxysterols of nonenzymatic origin, except for triol (Fig. 5). Similarly, a moderate inverse correlation was found between N3 duration and the enzyme-generated 27OHC but not for

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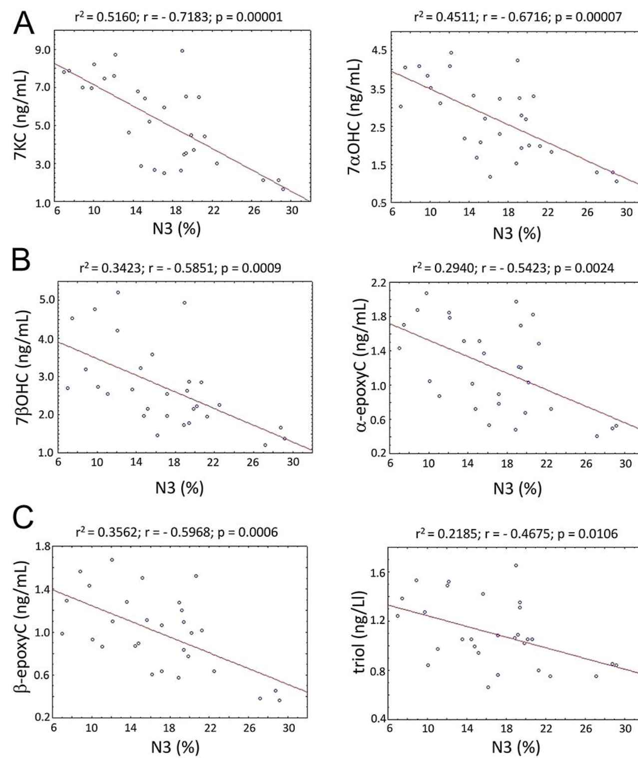

Figure 5

(A, B, C) Correlation analyses conducted to study the relationship between the NREM stage 3 (N3) sleep phase and the individual oxysterols of nonenzymatic origin. Pearson's correlation test was used. N3%, percentage of N3 sleep phase; 7αOHC, 7α-hydroxycholesterol; 7βOHC, 7β-hydroxycholesterol; 7KC, 7-ketocholesterol; α-epoxyC, α-epoxycholesterol; β-epoxyC, β-epoxycholesterol; triol, cholestane-3β,5α,6β-triol; *r*, Pearson correlation coefficient; r^2, coefficient of determination; and *P*, significance value.

24OHC (Fig. 6). Cholesterol and its three measured precursors showed an inverse correlation from modest to moderate degree with N3 duration (Fig. 7).

The data achieved from the correlation analyses for all tested oxysterols, cholesterol and its precursors and the adopted sleep biomarkers other than N3 are comprehensively reported in Supplementary Table 2S. In almost all cases, poor correlations were obtained, with a noteworthy exception, namely, a good inverse correlation between 7KC and the sleep efficiency marker (r = −0.6513).

Discussion

The occurrence of oxidative stress in AD (Cutler *et al.* 2004, Cioffi *et al.* 2019), as well as in NADs (Phan *et al.* 2020, Lyu *et al.* 2024), has been widely recognized but rarely investigated in combination with altered cholesterol metabolism and hypercholesterolemia,

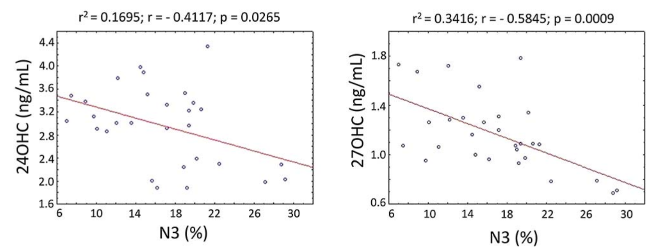

Figure 6

Correlation analyses conducted to study the relationship between the N3 sleep phase and 24-hydroxycholesterol and 27-hydroxycholesterol, oxysterols of enzymatic origin. Pearson's correlation test was used. N3%, percentage of N3 sleep phase; 24OHC, 24-hydroxycholesterol; 27OHC, 27-hydroxycholesterol; *r*, Pearson correlation coefficient; r^2, coefficient of determination; and *P*, significance value.

which are frequently associated with both AD (Anstey *et al.* 2017) and NADs (Wang *et al.* 2020) and/or in combination with the consistent sleep disorders of AD (Sani *et al.* 2019, Uddin *et al.* 2020) and NADs (Wennberg *et al.* 2017, Sani *et al.* 2019).

The association of oxidative stress with an increased deposition of cholesterol in the vulnerable areas of the AD brain was first described by Cutler and coworkers (Cutler *et al.* 2004), but deposition of cholesterol oxidation products in the brain at that time was not investigated. Only more recently, quantitative measurements performed in autoptic samples from AD brains with different degrees of pathology as classified by Braak staging demonstrated the accumulation of several

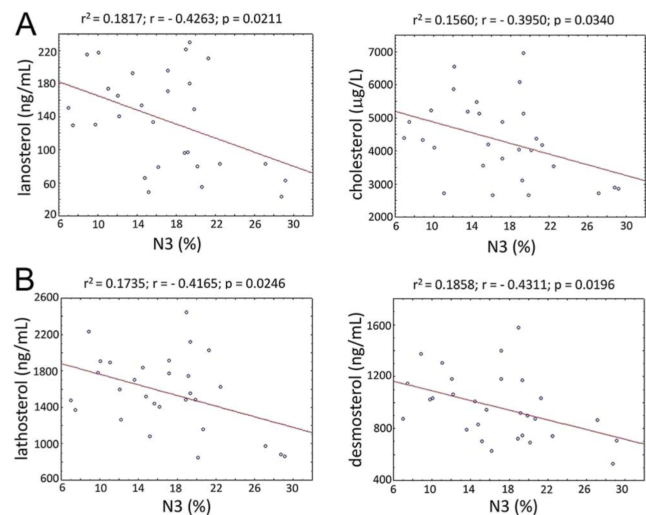

Figure 7

(A, B) Correlation analyses conducted to study the relationship between the N3 sleep phase and cholesterol and its main steroid precursors. Pearson's correlation test was used. N3%, percentage of N3 sleep phase; *r*, Pearson correlation coefficient; r^2, coefficient of determination; and *P*, significance value.

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oxysterols of nonenzymatic origin, not simply that of the commonly detected and investigated 27OHC (Testa *et al.* 2016). Preclinical studies on sleep deficiency have indicated its association with oxidative stress and the main molecular biomarkers of AD (Lv *et al.* 2022, Beiyu *et al.* 2024). In this pilot study, we correlated the quantification of oxidative stress and altered cholesterol metabolism with the PSG investigation in a relatively small number of patients with AD, NAD and patients without evidence of neurodegenerative or inflammatory diseases. It must be underlined that PSG is not a standard procedure in AD and NAD management; thus, specific expertise and structures are not always present in neurology and geriatric units.

The accumulation of free cholesterol in excess was demonstrated years ago to induce neuronal apoptosis (Zhou *et al.* 2012), a toxic action recently confirmed in a different setting as induced by reactive oxygen species-mediated signaling (Li *et al.* 2020). Much stronger pro-oxidant, pro-apoptotic and pro-inflammatory properties compared with those observed for free cholesterol have been consistently reported for several oxysterols in pathological amounts (Poli *et al.* 2013, de Freitas *et al.* 2021), being those of nonenzymatic origin, likely the most toxic ones (Vejux *et al.* 2020). These biochemical properties certainly allow oxysterols to provide a significant contribution to both initiation and especially progression of brain degenerative disease processes. Of note, brain C such as those affecting the patients in this study as internal control (Supplementary Table 2, see sectiion on Supplementary materials given at the end of the article) did not show evident changes either in cholesterol metabolism or in the CSF levels of oxysterols.

For all the enzymatic and nonenzymatic oxysterols quantified, we observed a marked increase in the CSF of patients with degenerative dementia, both AD and NAD, compared with the concentrations in the CSF collected from patients with nondegenerative disease, and striking was the evidence of a much higher increase in the oxysterols of autoxidation origin, hence markers of oxidative stress, all but β-epoxyC, in the CSF of AD compared with NAD patients (Fig. 1 and Table 1). On the contrary, the CSF concentrations of 24OHC and 27OHC did not show any statistically significant difference between AD and NAD patients (Fig. 2 and Table 1). Thus, among the oxysterols of autoxidation origin that can be measured in the CSF, there are certainly reliable candidate biomarkers of oxidative stress that might further help in elucidating molecular and clinical differences of AD and NAD dementias. Still, with regard to the measurement of oxidative stress in the CSF, the old parameter malondialdehyde did not prove to be useful at all, at least under the clinical conditions considered in this study (Fig. 2).

Moreover, since oxysterols are cholesterol oxidation products, their pathological increase in the CSF automatically highlights a derangement of cholesterol metabolism, also proved in AD and NAD patients by the concomitant increase of this steroid in the CSF as well as that of lanosterol and the intermediates in the Kandutsch–Russell pathway (lathosterol) and Bloch pathway (desmosterol) of cholesterol biosynthesis in the brain (Fig. 3 and Table 1). As for oxysterols stemmed from cholesterol autoxidation, CSF levels of cholesterol and desmosterol might be further useful indices to be considered in the clinical pathology of dementias. At least the indication stemming from this pilot study is that the increased synthesis of cholesterol observed in the NAD group and the significantly higher level in the AD group are fostered by a more active Bloch pathway.

The association of oxidative stress and cholesterol metabolism derangement in degenerative dementias could be hardly better supported than by the evidence that cholesterol metabolites deriving from its autoxidation strengthen and amplify the redox imbalance consistently occurring in those neurodegenerative disease processes.

Thus, oxysterols' increase in CSF, cholesterol dysmetabolism and polysomnographic data achieved in the three groups of patients were all considered together by means of correlation analyses. Out of the four selected markers of sleep disruption in the PSG analyses performed in the three groups of patients, only N3% showed a statistically significant difference, namely, a reduction, in AD and NAD groups compared with the patients with C (Fig. 4) and indeed the correlation analyses involving the N3 values provided interesting results. In fact, the most impressive and original finding of the present study was the strongly inverse correlation observed between the CSF level of some oxysterols of autoxidation origin, in particular 7KC and 7αOHC, reliable markers of oxidative stress and cholesterol dysmetabolism and the percent of N3, independent of sleep-disordered breathing (Fig. 5).

These results appear to indicate that the higher the CSF concentration of 7KC and 7αOHC and also of 7βOHC, the lower is the N3 phase of the sleep. The relatively highest CSF amounts of these three oxysterols were recovered in the patients with AD (Table 1). However, a reduction of the N3 duration implies reduced functionality (flow) of the glymphatic system (Hablitz & Nedergaard 2021, Voumvourakis *et al.* 2023). Thus, the increase in CSF levels of 7KC and 7αOHC would likely be associated with a reduced glymphatic flow.

Indeed, a reduced efficiency of the glymphatic system in removing waste products and noxious compounds would promote the accumulation of toxic compounds including oxysterols stemming from the brain into the CSF. The pro-inflammatory and pro-oxidant properties of 7KC and 7βOHC are unanimously recognized (Nury *et al.* 2021), being expressed by 7αOHC as well, while to a minor extent (Iaia *et al.* 2022). A delayed removal of oxysterols from the brain, because of a reduced glymphatic flow, will inevitably lead to their abnormal

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pile-up with a consequent robust contribution to the amplification of the oxidative and neuroinflammatory conditions. Moreover, the latter events would generate further oxysterols of nonenzymatic origin, favored by the increased cholesterol synthesis in the brain (Cutler *et al.* 2004). This is a dangerous vicious cycle that might contribute to worsening of the pathological process occurring in degenerative dementias, particularly in AD, the latter characterized by the highest CSF accumulation of cholesterol autoxidation-derived oxysterols.

Thus, if, on the one hand, the N3 sleep disruption occurring in AD, and to a minor extent in NAD patients, appears to significantly contribute to the engulfment of the oxysterols' flow in the CSF, on the other hand, it cannot be excluded that the derangement of cholesterol metabolism, occurring early and often preceding the neurodegeneration (Anstey *et al.* 2008, 2017), might contribute to generating and promoting the sleep disorders associated with dementia. The mechanisms through which oxidative stress and neuroinflammation could induce sleep abnormalities have to be elucidated yet, so are still matter of fascinating research.

The main limitation of the investigation reported here is certainly the relatively low number of recruited patients, especially of patients without nondegenerative diseases, so the research must be taken as a small case feasibility study. Indeed, ethically limited are the chances to obtain CSF samples from patients with nondegenerative diseases. Moreover, only a few neurological units combine clinical pathology tests with polysomnographic analyses in patients with dementia. In this relation, the participation of some of these units will be pursued in the future, while the recruitment of suitable patients at our center will continue. In this relation, the average reduction of N3% was more evident in the AD subgroup in comparison with the average reduction of N3% in the NAD subgroup, but the difference did not reach the statistical significance threshold (Fig. 4 and Table 1), further indicating the need to expand the two subgroups.

Another limitation is that the average age of the recruited patients with nondegenerative diseases was significantly lower than that of AD and NAD patients (Supplementary Table 1). Even if the statistical difference in the CSF oxysterol content and N3% sleep phase between AD and NAD in one group and patients in the other group were consistently very strong, the similar age in comparing the three subgroups of recruited patients is another task to be fulfilled, even if obviously uneasy.

The present small-scale study provides some useful suggestions: i) the measurement of defined oxysterols of nonenzymatic origin in the CSF as highly reliable biomarkers of oxidative stress and cholesterol metabolism disruption at the same time, ii) the combination of clinical pathology analyses with polysomnographic tests, in particular the measurement of N3, in the evaluation of neurodegenerative diseases, iii) the potential combination of oxysterol biomarkers with the evaluation of the glymphatic system's dynamics and changes by noninvasive procedures of magnetic resonance imaging (MRI) and iv) the vicious cycle between sleep disorders and oxysterol accumulation in the brain as a likely mechanism of disease amplification and progression.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REM-24-0013.

Declaration of interest

GP and VL are members of the Editorial Board of Redox Experimental Medicine. They were not involved in the review or editorial process for this paper on which they are listed as authors. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

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Author contribution statement

Lu P, Lo P, G P and A M conceived and supervised the overall study; Lu P, S C and G P wrote the manuscript; S C, E P, A M and S M performed all the molecular biology exper`iments; Lo P and R C performed the PSG tests; V L and D T performed the quantitative measurements of oxysterols; and Lu P, Lo P, V L, G P and A M and provided significant contribution to the discussion of the results.

Ethics approval consent to participate

This study involving human participants was reviewed and approved by IRCCS Istituto Auxologico Italiano Ethics Committee. The study was conducted in accordance with the Declaration of Helsinki. All participants gave their informed consent.

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