Cerebrospinal Fluid 7-Ketocholesterol Level Is Associated With Amyloid-β42 and White Matter Microstructure in Cognitively Healthy Adults

Running head**:** Oxysterols, $A\beta_{42}$, and white matter microstructure

Ane Iriondo[a], Maite García-Sebastian[a], Arantzazu Arrospide[b], Maria Arriba[a], Sara Aurtenetxe[a], Myriam Barandiaran[a], Montserrat Clerigue[a], Mirian Ecay-Torres[a], Ainara Estanga[a], Alazne Gabilondo[a], Andrea Izagirre[a], Jon Saldias[a], Mikel Tainta[a], Jorge Villanua[a], Kaj Blennow[c,d], Henrik Zetterberg[c,d,e,f], Javier Mar[b], Beatriz Abad-García[g], Irundika HK Dias[h], Felix M. Goñi[i], Pablo Martínez-Lage[a]

[a] Center for Research and Advanced Therapies. CITA-Alzheimer Foundation. Mikeletegi 71, 20009 Donostia-San Sebastian, Gipuzkoa, Spain

[b] Gipuzkoa Primary Care – Integrated Health Care Organizations Research Unit. Alto Deba Integrated Health Care Organisation, Nafarroa Hiribidea, 16, 20500 Arrasate, Gipuzkoa, Spain and Biodonostia Health Research Institute, Paseo Doctor Begiristain, s/n, 20014 Donostia - San Sebastian, Spain

[c] Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at University of Gothenburg, Mölndal, Sweden

[d] Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden

[e] Department of Neurodegenerative Disease, UCL Institute of Neurology, London, United Kingdom

[f] UK Dementia Research Institute at UCL, London, United Kingdom

[g] Central Analysis Service, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Bizkaia, Spain

[h] Aston Medical Research Institute, Aston Medical School, Aston University, Birmingham, B4 7ET, UK

[i] Departamento de Bioquímica, University of the Basque Country (UPV/EHU) and Instituto Biofisika (CSIC, UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Bizkaia, Spain

Corresponding Author:
Pablo Martinez-Lage
Fundación CITA-Alzheimer Fundazioa
Parque Científico tecnológico de Gipuzkoa, S.A.
Mikeletegi 71, bajo 20009 Donostia-San Sebastian (Gipuzkoa), Spain
Phone: 0034 943.021.792
pmlage@cita-alzheimer.org

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ABSTRACT

Background: Abnormal cholesterol metabolism changes the neuronal membrane and may promote amyloidogenesis. Oxysterols in cerebrospinal fluid (CSF) are related to Alzheimer's disease (AD) biomarkers in mild cognitive impairment and dementia. Cholesterol turnover is important for axonal and white matter (WM) microstructure maintenance.

Objective: We aim to demonstrate that the association of oxysterols, AD biomarkers, and WM microstructure occurs early in asymptomatic individuals.

Methods: We studied the association of inter-individual variability of CSF 24-hydroxycholesterol (24-OHC), 27-hydroxycholesterol (27-OHC), 7-ketocholesterol (7-KC), 7β-hydroxycholesterol (7β-OHC), amyloid-β42 (Aβ42), total-tau (t-tau), phosphorylated-tau (p-tau), neurofilament (NfL), and WM microstructure using diffusion tensor imaging, generalized linear models and moderation/mediation analyses in 153 healthy adults.

Results: Higher 7-KC levels were related to lower Aβ42, indicative of greater AD pathology ($p = 0.041$) . Higher 7-KC levels were related to lower fractional anisotropy (FA) and higher mean (MD), axial (AxD), and radial (RD) diffusivity. 7-KC modulated the association between AxD and NfL in the corpus callosum splenium ($B = 39.39$, $p = 0.017$), genu ($B = 68.64$, $p = 0.000$), and fornix ($B = 10.97$, $p = 0.000$). Lower Aβ42 levels were associated to lower FA and higher MD, AxD, and RD in the fornix, corpus callosum, inferior longitudinal fasciculus, and hippocampus. The association between AxD and Aβ42 was moderated by 7K-C ($p = 0.048$).

Conclusion: This study adds clinical evidence to support the role of 7K-C on axonal integrity and the involvement of cholesterol metabolism in the Aβ42 generation process.

INTRODUCTION

Oxysterols have been proposed as the link between brain cholesterol metabolism and Alzheimer's disease (AD) [1]. In AD, oxidative stress enhances the formation of oxysterols, which in turn increase pro-inflammatory mediator production and exacerbate neuronal damage, connecting all these processes in a vicious circle [2–4]. Moreover, oxysterols play a fundamental role in APP-processing and β-amyloid$_{1-42}$ (Aβ_{42}) generation as they induce changes in cell membrane dynamics [5] that are related to the amyloidogenic processing in lipid rafts [6,7]. While experimental work suggests that oxysterols contribute to amyloidogenesis [6–8], human studies have not shown an association between circulating oxysterols and CSF Aβ_{42} levels [9]. Higher 24-hydroxycholesterol (24OH-C) and 27-hidroxycholesterol (27OH-C), have been related to higher CSF total-tau (t-tau) and phosphorylated-tau (p-tau) levels in MCI and AD, but no in healthy people [10,11].

To analyze the axolemma and myelin integrity, which are highly dependent on cholesterol metabolism and turnover, the assessment of white matter (WM) microstructure using DTI-MRI, is a valuable in vivo neuroimaging technique [12]. Tensor-derived measurements include fractional anisotropy (FA) and radial, mean, and axial diffusivities (RD, MD, AxD) [13]. Despite the importance of cholesterol metabolism in the axolemma and myelin, few studies have analyzed the effect of cholesterol mis-metabolism on the microstructure of WM [14–16], and to our knowledge there is no previous work investigating the effect of oxysterols on WM microstructure. It is well established that patients with AD or mild cognitive impairment (MCI) show lower FA and higher MD, AxD and RD values especially in areas related to cognition, such as the superior and inferior longitudinal fasciculus, hippocampus or corpus callosum [17,18]. Moreover other studies have shown WM microstructure changes in cognitively healthy

people with positive amyloid biomarkers, especially in the fornix and the uncinate fascicle [19,20].

To better understand the relationship between oxysterol homeostasis and neuronal integrity, we aimed to examine the association of CSF oxysterols, AD CSF biomarkers and WM microstructure, especially in regions associated to cognitive performance. For the present study we focus on the two major oxysterols synthetized enzymatically, 24-hidroxycolesterol (24OH-C) and 27-hidroxycholesterol (27OH-C); and two synthetized non-enzymatically, 7β-hydroxycholesterol (7βOH-C) and 7-ketocholesterol (7K-C). We hypothesized that inter-individual variability of CSF oxysterols is related to changes in DTI indexes and is associated to lower $A\beta_{42}$ and/or higher t-tau o p-tau levels. Furthermore, to investigate the role of inflammation and the occurrence of axonal damage, we have also looked at YKL-40 and neurofilament light (NfL).

MATERIALS AND METHODS

Subjects

This study is a cross-sectional analysis of baseline data from the Gipuzkoa Alzheimer Project (GAP). The GAP study is an ongoing longitudinal study on preclinical and prodromal phases of AD. A cohort of 410 non-demented volunteers (aged 40-80) was recruited from the community through local media advertisements and presentations at the local Alzheimer Association. Baseline visits took place between June 2011 and January 2013. Exclusion criteria were dementia and any significant neurologic, systemic, or psychiatric disorder causing cognitive impairment. All participants completed a comprehensive clinical and neuropsychological evaluation, anthropometric and cardiovascular risk assessment, blood workup, apolipoprotein E (*APOE*) genotyping for the ε2/ε3/ε4 polymorphism, as well as brain magnetic resonance imaging

(MRI). Lumbar puncture for CSF biomarkers was optional. For the present study was selected GAP participants deemed clinically normal based on a global Clinical Dementia Rating (CDR) score of 0 [21] with available CSF samples (n=153). For the white matter microstructure analyses, 26 participants were excluded due to incidental findings and/or present of MRI artifacts identified by visual inspection and noted non-usable for this study.

The study was approved by the local Ethics and Clinical Research Committee, and all subjects gave written informed consent to participate.

Magnetic Resonance Imaging (MRI)

Data acquisition

Whole brain scans were obtained using a Siemens 3T Magnetom TrioTim scanner (Siemens, Erlangen, Germany) in combination with a 32-channel head coil. To increase the inter-subject homogeneity of the image acquisition, the AutoAlign Head LS software tool (Siemens) was used. Diffusion-weighted images were obtained using an echo planar imaging (EPI) sequence with the following specifications: TR 9300 ms, TE 92 ms, voxel size: 1.7-mm isotropic, 71 consecutive slices, acquisition matrix 122×122 (FOV 208 mm), 6/8 partial Fourier, 64 diffusion directions with b-value 1000 s/mm2, and one image with no diffusion weighting. The bandwidth was 1640 Hz/pixel.

Image processing and analyses

First, DICOM images were converted into 4D compressed NIfTI files and diffusion gradient directions were extracted with dcm2nii (part of MRIcron package, http://www.mccauslandcenter.sc.edu/mricro/mricron/). Then 4D brain volumes were processed and analysed by Tract-Based Spatial Statistics (TBSS), part of the FSL toolbox (FMRIB Software Library, Version 5.0.5; FMRIB, Oxford, United Kingdom) [22–24]. After image conversion, scalar DTI maps (FA, MD, AxD, and RD) were created

by fitting a tensor model to the raw diffusion data using FDT (FMRIB's Diffusion Toolbox). Then FA images were brain-extracted using Brain Extraction Tool (BET) [25], with b=0 as the reference volume. All subjects FA data were then aligned into a study-specific FA template, using the nonlinear registration tool FNIRT [26], which uses a b-spline representation of the registration warp field [27]. The resulting warp-fields were then applied to MD, AxD and RD images. Due to the fact that in this study the subjects are middle to older age (40-77 years), we created a study-specific FA template instead of using the standard template (FMRIB58_FA) provided by FSL software, which is based on 58 subjects from 20 to 50 years. Before voxel-wise statistical analyses, all subject´s FA data were obtained and projected onto a mean FA tract skeleton, which represents the centres of all tracts common to the group [28].

CSF biomarker measurements

CSF was obtained and collected following international consensus recommendations as described previously [29]. Samples were aliquoted and stored in polypropylene tubes at -80ºC and shipped on dry ice to the Clinical Neurochemistry Laboratory in Gothenburg for analysis. Biomarker concentrations were measured by board-certified laboratory technicians using commercial assays as previously described[30] (MSD: $A\beta_{42}$, $A\beta_{40}$; Fujirebio Europe INNOTEST: t-tau and p-tau; R&D: YKL-40; and UmanDiagnostics: NfL). As provided by the laboratory participants with $A\beta_{42}/A\beta_{40} < 0.08$ pg/mL, were considered amyloid positive (Aβ+).

Quantification of CSF oxysterols by LC-MS/MS

Human CSF samples (400 µl) spiked with internal standards (1 ng 24-OHCd7, 0.25 ng 25-OHCd6, 4 ng 27-OHCd6, 0.5 ng 7β-OHCd7, 15 ng 7-KCd7) was mixed with 1600 µl ice-cold methanol containing 4 mg/ml butyl hydroxytoluene (BHT) and incubated for 10 min in ice. Samples were then centrifuged (14,000 xg at 4°C for 10 min) and the

methanolic supernatant was diluted with acidified water up to 12.5 % of methanol. Oxysterol enrichment and quantification was performed as described by Dias et al 2018[31]. Briefly, oxysterols were extracted using Oasis HLB Prime cartridges (bed wt. 30 mg, 1 ml, 96-well) and analysed by liquid chromatography UltiMate 3000 HPLC (Dionex, Thermo Scientific Ltd., Hemel Hempstead, UK) on-line coupled to the ESI-QqLIT-MS/MS mass spectrometer (QTrap 5500, AB Sciex, Warrington, UK). Multiple reaction monitoring with transitions of 367.2/161 for 24-OHC, 367.4/ 147 for 25-OHC, 385.4/161 for 27-OHC, 385.4/81 for 7β-OHC and 401.4/95 for 7-KC were used to collect data. Data were examined using Analyst Software 1.7.2 (AB Sciex, Warrington, UK).

***APOE* genotype**

APOE genotype was determined using 1-stage polymerase chain reaction as previously described [32]. Participants were classified as *APOE* ε4 carriers (APOE4+) if they had at least one ε4 allele, or as non-carriers (APOE4-).

White matter hyperintensities

Individual global white matter hyperintensity (WMH) was semiquantified by the Fazekas scale by an experienced neuroradiologist on fluid attenuation inversion recovery MRI sequences. Fazekas scale ranges between 0 and 3 (0= no WMH; 1 = focal/punctate lesions; 2 = beginning confluent lesions; 3 = confluent-diffuse lesions) [33].

Statistical Analysis

To assess the appropriateness of parametric statistics, the Kolmogorov-Smirnov test was used to examine the normality of oxysterol distribution. Frequency distributions were calculated for categorical variables, and means (standard deviations) or median ($percentile_{25}$-$percentile_{75}$) were calculated for continuous variables.

<u>Oxysterols and AD biomarkers</u>

Separate generalized linear models (GLM) were conducted to explore the association between oxysterol measured by 24OH-C, 27OH-C, 7K-C, 7βOH-C and CSF biomarkers ($A\beta_{42}$, t-tau, p-tau) adjusted by age, sex and *APOE* genotype. If a significant association was found, we examined the role of YKL40 including this variable in a new regression model.

White matter microstructure

All DTI indexes, were correlated with age, sex and WMH, therefore all analysis were adjusted for these confounders. To test the association between oxysterols and WM microstructure as indexed by the DTI measurements (FA, MD, RD, AxD), whole-brain voxel-wise statistical analyses were performed with a nonparametric permutation inference tool (randomise, part of the FSL toolbox). For these analyses 5000 permutations were generated and Threshold-Free Cluster Enhancement (TFCE) was conducted as a thresholding option [34]. All the resulting statistical maps were corrected for multiple comparisons with family wise error (FWE), thresholded at $p<0.05$ and only clusters with at least 100 contiguous voxels were defined as significant clusters. Resulting significant clusters were labelled using the atlas available through FSL toolbox (ICBM-DTI-81 white matter labels atlas).

Based on previous results linking WM microstructure with cognitive performance [17,35], special attention was focused on the following regions: corpus callosum, inferior and superior longitudinal fasciculus, cingulate gyrus, hippocampus, uncinate fasciculus, fornix (column+body) and fornix cres/stria terminalis These regions were labelled using the atlas available through FSL toolbox (ICBM-DTI-81 white matter labels atlas).

If an oxysterol showed a significant association with an AD biomarker and a DTI index, the effect of the biomarker on the DTI index was analysed in the voxels where the oxysterol had shown a significant effect. Individual mean values for DTI indexes were

extracted in regional clusters that were significantly associated with oxysterols according to TBSS analysis (FEW-corrected $p<0.05$). For these analyses, GLMs were conducted. The dependent variable was the mean of the DTI measures of each region and the predictor was the biomarker. The DTI indexes were multiplied by 1000 in the analyses [36].

To better understand the association between oxysterols, biomarkers and WM microstructure in areas related to cognition, GLMs were designed for the DTI indexes obtained from each region where the oxysterol and biomarkers showed a significant effect. The biomarker was the dependent variable and oxysterol, DTI index and the interaction between both were predictive variables. Mediation and moderation analyses were performed to better understand the associations found between oxysterols, AD biomarkers and cognitive regions WM microstructure. Moderation is shown up by a significant interaction effect.

Finally, we conducted new GLMs to study the effect of the interaction between oxysterols and DTI indexes and between biomarkers and DTI indexes on CSF NfL levels, as a biomarker of axonal damage. Mediation and moderation analyses were performed to better understand the associations found between oxysterols, AD biomarkers, cognitive regions DTI indexes and NfL. All models were controlled by sex, age, WMH and *APOE* genotype.

Statistical analyses were conducted in SPSS version 20. For mediation and moderation analysis we applied the PROCESS macro for SPSS (version 3) by Andrew F.Hayes (http://www.afhayes.com).

RESULTS

Descriptive data for the whole sample (n = 153, CDR score= 0) are shown in Table 1. Participant age range was 40-75, 67 (43.8%) were women and mean MMSE score was 29.

Oxysterols and CSF biomarkers for AD pathology

To study the association between oxysterol levels and CSF AD biomarkers, GLMs were conducted among 153 healthy participants controlling for age, gender and *APOE* genotype. 7K-C was associated with $A\beta_{42}$ (B = -0.20; p = 0.041) (Table 2). There were no effects of 7K-C on t-tau or p-tau. The other oxysterols, 24OH-C, 27OH-C, 7βOH-C, did not show any significant effect on any CSF biomarker.

As $A\beta_{42}$ was significantly correlated with YKL40 (r_s = 0.244; p = 0.003) and 7K-C is related to apoptosis and inflammation [37] we introduced YKL40 in the model. YKL40 (B = 0.001; p = 0.000) and 7K-C (B= -0.19; p = 0.044) showed a significant effect on $A\beta_{42}$. . The interaction between YKL40 and 7K-C, was not significant (B= -5.88E-7; p = 0.812).

Oxysterols and white matter microstructure

To study the correlation between oxysterols and WM microstructure, voxel-wise correlational analysis was conducted among 127 healthy participants controlling for age, gender and white matter hyperintensities. Descriptive data for the subsample with available MRI included significantly more women than the all sample (p = 0.005) (Table 1).

TBSS analysis detected a significant negative association between 7K-C and FA (Figure 1A). Most of these voxels were located in the corpus callosum. Significant associations were also seen in the following regions: left anterior and right posterior corona radiata, left external capsule, right hippocampus and right superior longitudinal fascicule. We did not observe significant positive association between 7K-C and FA. Analyses also revealed a significant positive association correlation between 7K-C and MD (Figure 1B), AxD (Figure 1C) and RD (Figure 1D) distributed throughout the whole brain WM in both hemispheres. We did not observe significant negative association between 7K-C

and MD, AxD or RD. Table 3 shows the distribution and proportion of voxels where a significant association between 7K-C and each DTI measure was detected in every cognitive region.

There was no significant effect for 24OH-C, 27OH-C and 7βOH-C in any of the DTI measurements.

Oxysterols, CSF biomarkers for AD pathology and cognitive regions white matter microstructure

As 7K-C had a significant effect on $A\beta_{42,}$ the association between $A\beta_{42}$ and DTI indexes of WM microstructure was analysed in the voxels where 7K-C showed a significant effect on each index (Table 3). GLMs were adjusted for age, gender, WMH and *APOE* genotype.

$A\beta_{42}$ had a positive effect on FA of the splenium of the corpus callosum and AxD of right hippocampus, and a negative effect on the MD of the body of the corpus callosum, fornix (column+body) and left inferior longitudinal fasciculus; on AxD of the genu and splenium of the corpus callosum and fornix (Column+body); and on RD of the fornix (column+body) (Figure 2). The βA+ group (n=25) had significantly higher MD, AxD and RD values in the fornix (column+body) than βA- group (n=126) (MD: βA+, *median* = 1.75; βA-, *median* = 1.42; $p = 0.001$ / AxD: βA+, *median* = 2.32; βA-, *median* = 2.03; $p = 0.003$ / RD: βA+, *median* = 1.50; βA-, *median* = 1.11; $p = 0.002$) (Figure 2).

To explore the association between 7K-C, $A\beta_{42}$ and WM microstructure, the effect of 7K-C and its interaction with the WM microstructure indexes (FA*7K-C; MD*7K-C; AxD*7K-C; RD*7K-C) on the level of $A\beta_{42}$ was studied in the areas related to cognitive performance where the 7K-C and $A\beta_{42}$ showed significant effect. Higher MD, AxD and RD of the fornix, and higher AxD of the splenium and genu of the corpus callosum, right hippocampus and left inferior longitudinal fasciculus were related to

lower $A\beta_{42}$ (Table 4). In these model 7K-C and the interactions between 7K-C and DTI measures were not significant. In the left inferior longitudinal fasciculus model, AxD, 7K-C and 7K-C*AxD interaction showed a significant effect. Moderation analyses showed that the relationship between AxD and $A\beta_{42}$ was moderated by 7K-C level (B = 3.87, p = 0.048). When YKL40 was included in the analyses, the moderation effect of 7K-C was not significant (p = 0.082).

Oxysterols, CSF AD biomarkers, axial diffusivity and neurofilament,

As AxD is related to axonal damage, the effect of the 7K-C*AxD interaction, and the $A\beta_{42}$*AxD interaction on NfL for areas related to cognition where 7K-C and $A\beta_{42}$ had significant effect were analyzed in separate GLMs. The 7K-C*AxD interaction showed a significant effect on NfL in the fornix and in the splenium and genu of the corpus callosum (Table 5). In these regions, the relationship between AxD and NfL was moderated by 7K-C (Fornix: B = 1.53, p = 0.000; splenium: B = 5.51, p = 0.017; Genu: B = 9.61, p = 0.000). The $A\beta_{42}$*AxD showed no significant effect on NfL in any region.

DISCUSSION

We have investigated the association between oxysterols, CSF AD biomarkers and WM microstructure in cognitive regions in a population of cognitively healthy middle-aged subjects. To our knowledge, this might be the first study on the effect of oxysterols on WM microstructure and the first to report that higher 7K-C levels are related to lower $A\beta_{42}$. 7K-C had a negative effect on FA and a positive effect on MD, AxD and RD in multiple cognitive regions. Moreover, 7K-C modulated the association between AxD and axonal loss, as measured by NfL, in the splenium and genu of the corpus callosum and fornix. Interestingly, in the voxels where 7K-C showed a significant effect on FA, MD, AxD and RD, $A\beta_{42}$ also showed positive association with FA and negative effects

on MD, AxD and RD in the fornix, corpus callosum, inferior longitudinal fasciculus and hippocampus. Furthermore subjects who were βA+ had significantly higher MD, AxD and RD values in the fornix than the βA- group.

Previous case-control studies on CSF oxysterol levels have reported that 24OH-C and 27OH-C levels are higher in patients with AD or MCI [10,11,38]. Other studies, analyzing patients with AD or MCI and controls al together, have shown positive correlations between 24OH-C CSF concentrations and t-tau and p-tau levels, but not with $A\beta_{42}$ [10,39]. However these correlations were significant only in the AD or MCI group, and no in healthy people. Popp and colleagues described a positive correlation between 24OH-C and sAPPα and sAPPβ in patients with AD, but no with $A\beta_{42}$ [40]. Our results confirm the absence of association between 24OH-C and 27OH-C and CSF AD biomarkers in cognitively healthy adults. This suggests that if 24OH-C and 27OH-C are involved in AD pathophysiology this would not represent an early event. Although 24-OHC and 27-OHC are the oxysterols most widely considered to be potentially implicated in AD pathogenesis, the possible involvement of oxysterols resulting from cholesterol autoxidation is now emerging. In fact, in a recent systematic analysis of oxysterols in post-mortem human AD brains, the level of some of the oxysterols deriving from cholesterol autoxidation were higher in subjects in the Braak I-II stages than in controls, and there were no differences in the levels of the two oxysterols of enzymatic origin [41]. 7K-C is a major product of reactive oxygen species (ROS)-caused oxidation of cholesterol [42]. We found that higher 7K-C levels were associated with lower $A\beta_{42}$. This result is in line with previous model lipid membrane studies reporting a relation between 7K-C and $A\beta_{42}$ [43,44]. In the study by Kim and colleagues, they observed that partial substitution of cholesterol with 7K-C in the model lipid membrane enhances $A\beta_{42}$ insertion into the lipid bilayer, by decreasing intramolecular cohesive interactions

[44]. In the same line, Phan and colleagues demonstrated that 7K-C renders lipid bilayer less condensed and more fluid than cholesterol, thus accelerating $A\beta_{42}$ association with the bilayer [6], specially the protofibrillar $A\beta_{42}$ [43]. Moreover, a recent model lipid membrane study demonstrated that cholesterol and 7K-C have different effects on membrane-mediated aggregation of $A\beta_{42}$. While cholesterol inhibited the nucleation step and accelerated fibrillar $A\beta_{42}$ growth, the partial substitution of membrane cholesterol with 7K-C slightly increased the nucleation fase and remarkably decreased fibril elongation [45]. The oligomers or protofibrillar $A\beta_{42}$ formed in the nucleation step are reportedly more toxic than soluble monomers and mature fibrils [46]. These papers suggested that cholesterol and 7K-C can modulate interaction of $A\beta_{42}$ with cell membranes by influencing the fibrillation of the peptide. Our work adds clinical evidence to support these experimental studies on the relation between 7K-C and $A\beta_{42}$. An in vitro study showed that 7K-C induced apoptosis in cells associated with increased ROS generation, ER stress and upregulated caspase-3/7 activity [37]. Thus, we hypothesized that neuroinflammation, as measured by YKL40 had an effect in the relation between 7K-C and $A\beta_{42}$. Our results demonstrated that both, 7K-C and YKL40, have a significant effect on $A\beta_{42,}$ but there is no interaction between 7K-C and YKL40, suggesting that both influence the amyloidogenic process by different pathways. Regarding WM microstructure, to our knowledge, this might be the first study investigating the effect of oxysterols on WM microstructure. We found that 7K-C had a negative effect on FA and a positive effect on MD, AxD and RD in multiple cognitive regions. Moreover, 7K-C modulates the relation between NfL, a marker of axonal damage [47], and AxD, the DTI index related to axonal damage [48]. 7K-C induces oxidative stress [37] which in turn induces activation of microglia and astrocytes with a consequent increase of pro-inflammatory mediator production and exacerbating

neuronal damage [2]. Our results suggest that the WM microstructure changes related to neuronal and axonal damage are related to altered cholesterol metabolism and increased membrane levels of 7K-C. With respect to $A\beta_{42}$, we found a relation between lower CSF $A\beta_{42}$ and lower FA and higher MD, AxD or RD in areas related to cognitive performance such as fornix, corpus callosum, inferior longitudinal fasciculus and hippocampus in those voxels where 7K-C showed a significant effect on the DTI indexes. Previous studies have shown lower FA and higher MD in patients with AD, especially in areas such as corpus callosum, fornix, superior and inferior longitudinal fasciculus or cingulum [17,18,49]. Similar changes in WM microstructure are also found in cognitively healthy persons with abnormal $A\beta_{42}$ levels, specially in the uncinate fasciculus and fornix [19,20]. These findings are in line with our results. In our study βA+ subjects have significantly higher MD, AxD and RD values in the fornix than the βA- group.

We have found a relation between $A\beta_{42}$, 7K-C and WM microstructure in cognitive regions. Axon integrity depends mainly on the axolemma structure which in turn depends on cholesterol turnover. Our work would suggest that cholesterol metabolism and integration of 7K-C in the axolemma could change the structure of the membrane, alter WM microstructure and modulate the amyloidogenic process.

The GAP study has allowed us to analyze a rigorously phenotyped cohort with strict clinical and cognitive criteria to exclude participants with cognitive impairment rigorously. However, the study must also be interpreted in the light of its limitations. The principal one is that DTI metrics are an indirect measure of WM microstructure and the influence of crossing fibers makes the interpretation more complicated in all DTI studies. However, the strength of the study is that participants performed a high resolution 3T-DWI with 65 directions which has allowed to realize robust estimates of

tensor derived-properties [50]. This is a cross-sectional study, which cannot characterize how the association between 7K-C, $A\beta_{42}$ and WM microstructure changes over time. Longitudinal follow-up data on the GAP study cohort are now being collected and will be the subject of further analysis to confirm these cross-sectional findings.

In conclusion, this study shows that interindividual variability of CSF 7K-C levels in healthy adults are associated with $A\beta_{42}$ levels and WM integrity in cognitive regions. Perhaps, for the first time, this study adds clinical evidence to support experimental studies on the potential effect of 7K-C in the $A\beta_{42}$ aggregation process and membrane structure. Furthermore, this study reports, possibly for the first time, the effect of 7K-C on the WM microstructure and its modulating effect on the relation between axonal damage and white matter microstructure, suggesting that WM microstructure changes related to 7K-C could be a mirror of the neuronal damage induced by 7K-C.

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Author contributions

AI and PML conception and design of the study; AI, MGS, MA, SA, MB, MC, MET, AE, AG, AIz., JS, MT, JV, KB, HZ, BAG, IHK, and PML acquisition of data; AI, AA, JM and PML analysis of data; AI, MGS, IHK, FMG and PML drafting the manuscript.

Potential conflicts of interest

HZ has served at scientific advisory boards for Roche Diagnostics, Samumed, CogRx and Wave, has given lectures in symposia sponsored by Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. The other authors declare no conflicts of interest.

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Figure legends

Figure 1: Association between 7K-C and white matter microstructure. Data adjusted for age, sex and white matter hyperintensities (family-wise error-corrected, thresholded at $p<0.05$, n=127). Results are shown as an overlay on the FSL standard T1 template brain (coordinates according to MINI152 template). Abbreviations: 7K-C, 7-ketocholesterol; L, left; R, right, FA, fractional anisotropy; MD, mean diffusivity; AxD, axial diffusivity; RD, radial diffusivity.

Figure 2: Association between $A\beta_{42}$ and white matter microstructure. Note: analyses were performed in the voxels were 7-ketocholesterol showed significant effect. Adjusted for age, sex, white matter hyperintensities and *APOE* genotype. Abbreviations: $A\beta_{42,}$ β-amyloid$_{1-42}$; FA, fractional anisotropy; MD, mean diffusivity; AxD, axial diffusivity; RD, radial diffusivity.

Tables

Table 1: Sample characteristics

Characteristic	All sample (n=153)	Sample with MRI (n=127)	p[a]
Age, years	57.09 (53.06 – 62.12)	56.85 (53.01-62.35)	0.664
Sex (% female)	67 (43.8)	78 (61.4)	**0.005**
Education, years	14 (11 – 17)	14.00 (11-17)	0.525
MMSE, score	29 (28 – 30)	29.00 (28-30)	0.719
APOE ε4 carrier	36 (23.5)	27 (21.3)	0.202
Fazekas scale, n (%)			0.953
0	86 (56.6)	71 (55.9)	
1	52 (34.2)	44 (34.6)	
2	9 (5.9)	8 (6.3)	
3	5 (3.3)	4 (3.1)	
24OH-C, ng/dl	12.47 (9.70-19.05)	12.38 (9.70-19.06)	0.973
27OH-C, ng/ml	2.28 (1.24)	2.29 (1.10)	0.924
7K-C, ng/dl	97.18 (45.99-174.66)	93.71 (45.40-146.12)	0.066
7βOH-C, ng/dl	19.46 (12.85-33.88)	19.65 (12.85-34.54)	0.915
$A\beta_{42}$, pg/ml	478.73 (152.22)	478.31 (154.02)	0.939
t-tau, pg/ml	268.40 (201.87-336.76)	270.87 (194.24-353.65)	0.451
p-tau, pg/ml	38.50 (30-46)	39.00 (30-47)	0.678
YKL40, pg/ml	121759.67 (92656.61-153900.96)	119796.16 (88460.09-153787.68)	0.536
NfL, pg/ml	501.86 (397.22-630.78)	501.86 (391.12-630.78)	0.730

Note. Significant effects are shown in bold. Mean (Standard Deviation) of measures, median (p25-p75) and in categorical variables, n (%). 24OH-C, 24-hidroxycholesterol; 27OH-C, 27-hidroxycholesterol; 7K-C, 7-ketocholesterol; 7βOH-C, 7β-hidroxycholesterol; MMSE, Mini Mental Status Examination; *APOE*, Apolipoprotein E; NfL, Neurofilament Light

a. Comparison between participants with and without MRI.

Table 2: Relationship between CSF oxysterols and AD CSF biomarkers

	$A\beta_{42}$		**t-tau**		**p-tau**	
	B (95%IC)	p	B (95%IC)	p	B (95%IC)	p
Model 1						
24OH-C	-0.91 (-3.31-1.49)	0.457	-0.19 (-1.82-1.45)	0.821	0.01 (-0.19-0.21)	0.939
Age	1.35 (-2.23-4.92)	0.460	6.76 (4.33-9.19)	**0.000**	0.77 (0.48-1.07)	**0.000**
Gender: F	-4.39 (-52.61-43.84)	0.858	-7.42 (-40.26-25.42)	0.658	0.08 (-3.89-4.06)	0.967
Gender: M	0[a]		0[a]		0[a]	
APOE4+	-52.10 (-108.91-4.70)	0.072	21.37 (-17.14-59.88)	0.277	2.58 (-2.07-7.23)	0.277
APOE4-	0[a]		0[a]		0[a]	
Model 2						
27OH-C	5.86 (-13.85-25.57)	0.560	1.98 (-11.43-15.39)	0.772	0.91 (-0.70-2.53)	0.266
Age	1.31 (-2.29-4.90)	0.476	6.78 (4.34-9.22)	**0.000**	0.80 (0.50-1.09)	**0.000**
Gender: F	-5.68 (-54.74-43.38)	0.820	-6.52 (-39.89-26.84)	0.702	0.06 (-3.96-4.09)	0.975
Gender: M	0[a]		0[a]		0[a]	
APOE4+	-54.15 (-112.04-3.74)	0.067	23.42 (-15.78-62.63)	0.242	2.82 (-1.89-7.54)	0.241
APOE4-	0[a]		0[a]		0[a]	
Model 3						
7K-C	-0.20 (-0.39--0.01)	**0.041**	-0.06 (-0.19-0.07)	0.338	-0.01 (-0.03-0.00)	0.170
Age	1.44 (-2.08-4.96)	0.422	6.79 (4.38-9.20)	**0.000**	0.79 (0.50-1.08)	**0.000**
Gender: F	-3.17 (-51.10-44.76)	0.897	-6.33 (-39.23-26.57)	0.706	0.17 (-3.80-4.14)	0.934
Gender: M	0[a]		0[a]		0[a]	
APOE4+	-54.87 (-111.25-1.51)	0.056	19.96 (-18.58-58.51)	0.310	2.42 (-2.23-7.06)	0.308
APOE4-	0[a]		0[a]		0[a]	
Model 4						
7β-C	0.44 (-0.43-1.32)	0.321	-0.42 (-1.00-0.16)	0.160	-0.05 (-0.12-0.02)	0.201
Age	0.91 (-2.68-4.49)	0.619	6.86 (4.44-9.29)	**0.000**	0.79 (0.49-1.08)	**0.000**
Gender: F	-6.39 (-55.54-42.76)	0.799	-9.26 (-42.55-24.04)	0.586	-0.18 (-4.21-3.85)	0.930
Gender: M	0[a]		0[a]		0[a]	
APOE4+	-55.91 (-113.10-1.29)	0.055	23.15 (-15.44-61.75)	0.240	2.75 (-1.91-7.41)	0.248
APOE4-	0[a]		0[a]		0[a]	

Note. Significant effects are shown in bold. B, beta coefficient; CI, confidence interval; APOE4+ = apolipoprotein E ε4 carrier, APOE4- = apolipoprotein E ε4 non-carrier; 24OH-C, 24-hidroxycholesterol; 27OH-C, 27-hidroxycholesterol; 7K-C, 7-ketocholesterol; 7βOH-C, 7β-hydroxycholesterol; $A\beta_{42}$ = β-amyloid$_{1-42}$; t-tau = total-tau; p-tau = phosphorylated tau

a. Set to zero because this parameter is redundant.

Table 3. Distribution and proportion of significant voxels for the association between 7K-C and cognitive regions DTI indexes.

Region (Nº of voxels)[a]		Number of significant voxels (%)[b]			
		FA	MD	AxD	RD
CC	Genu (8851)	429 (4.85)	470 (5.31)	98 (1.11)	2 (0.02)
	Body (13711)	247 (1.80)	658 (4.80)	0	21 (0.15)
	Splenium (12729)	223 (1.75)	524 (4.12)	116 (0.91)	387 (3.04)
Fornix	Column+body (659)	0	50 (7.59)	53 (8.04)	47 (7.13)
	Cres/stria terminalis Right (1124)	0	36 (3.20)	16 (1.42)	42 (3.74)
	Cres/stria terminalis Left (1125)	0	68 (6.04)	55 (4.89)	61 (5.42)
ILF	Right (2228)	0	298 (13.38)	231 (10.37)	255 (11.45)
	Left (2231)	0	258 (11.56)	240 (10.76)	35 (1.57)
SLF	Right (6607)	118 (1.79)	578 (8.75)	332 (5.02)	617(9.34)
	Left (6605)	0	447 (6.77)	4 (0.06)	397 (6.01)
GC	Right (2342)	0	31 (1.32)	0	0
	Left (2751)	16 (0.58)	42 (1.53)	0	0
Hippoc.	Right (1236)	239 (19.34)	229 (18.53)	28 (2.27)	247 (19.98)
	Left (1155)	0	120 (10.39)	0	0
FU	Right (380)	0	38 (10.00)	0	0
	Left (376)	0	0	0	0

Note. Number of significant voxels (FEW-corrected and $p<0.05$). CC, corpus callosum; ILF, inferior longitudinal fasciculus; SLF, superior longitudinal fasciculus; GC, gyrus cingulum; Hippoc., hippocampus; UF, uncinated fasciculus

a. In parenthesis, the number of voxels according to ICBM-DTI-81 white matter labels atlas

b. In parenthesis the proportion of significant voxels with respect to the total number of voxels in this area

Table 4: Effect of 7K-C and cognitive regions WM microstructure on $A\beta_{42}$ levels.

	FRNX (MD)		**FRNX (AxD)**		**FRNX (RD)**	
	B (95%IC)	p[a]	B (95%IC)	p[a]	B (95%IC)	p[a]
7K-C	-0.15 (-0.76-0.46)	0.635	-0.13 (-0.98-0.72)	0.771	-0.15 (-0.65-0.36)	0.575
MD	-121.19 (-207.72-34.66)	**0.006**				
7K-C*MD	0.02 (-0.29-0.34)	0.890				
AxD			-131.74 (-230.42-33.06)	**0.009**		
7K-C*AxD			0.01 (-0.34-0.36)	0.955		
RD					-112.62 (-192.35-32.89)	**0.006**
7K-C*RD					0.02 (-0.28-0.32)	0.884
	CC-S (FA)		**CC-S (AxD)**		**CC-B (MD)**	
	B (95%IC)	p[a]	B (95%IC)	p[a]	B (95%IC)	p[a]
7K-C	0.65 (-4.96-6.26)	0.820	-2.50 (-6.14-1.13)	0.177	0.80 (-2.61-4.21)	0.645
FA	1.09 (-0.39-2.57)	0.148				
7K-C*FA	-0.001 (-0.008-0.006)	0.796				
AxD			-472.22 (-900.19-44.26)	**0.031**		
7K-C*AxD			1.54 (-0.80-3.89)	0.198		
MD					-457.53 (-1498.56-583.51)	0.389
7K-C*MD					-1.198 (-5.651-3.254)	0.598
	CC-G (AxD)		**ILF L (AxD)**		**Hippoc. R (AxD)**	
	B (95%IC)	p[a]	B (95%IC)	p[a]	B (95%IC)	p[a]
7K-C	-0.31 (-4.16-3.54)	0.873	-5.42 (-10.49-0.35)	**0.036**	-0.02 (-1.17-1.13)	0.976
AD	-440.17 (-813.01-67.33)	**0.021**	-722.00 (-1287.77-156.23)	**0.012**	248.42 (57.91-438.94)	**0.011**
7K-C*AxD	0.13 (-2.30-2.56)	0.914	3.87 (0.17-7.58)	**0.040**	-0.23 (-1.35-0.89)	0.686

Note. Significant effects are shown in bold. B, beta coefficient; CI, confidence interval; $A\beta_{42}$ = .β-amyloid$_{1-42}$; CC-S, corpus callosum-splenium; CC-B, corpus callosum-body; CC-G, corpus-callosum-genu; ILF L, inferior longitudinal fasciculus left; FRNX, fornix (column+body); Hippoc. R, hippocampus right, FA, fractional anisotropy; MD, mean diffusivity; AxD, axial diffusivity; RD, radial diffusivity.

a. Generalized linear models adjusted for gender, age, white matter hyperintensities and *APOE* genotype

Table 5: Effect of 7K-C, $A\beta_{42}$ and cognitive regions axial diffusivity on NfL levels.

	FRNX		**CC-S**	
	B (95%IC)	p[a]	B (95%IC)	p[a]
Model 1				
AxD	-3.37 (-4.88--1.86)	**0.000**	-633.11 (-1425.09-158.87)	0.117
7K-C	-202.83 (-377.69--27.97)	**0.023**	-8.19 (-14.91--1.46)	**0.017**
AxD *7K-C	1.54 (0.92-2.15)	**0.000**	5.52 (1.17-9.86)	**0.013**
Model 2				
AxD	331.43 (-121.02-783.88)	0.151	-456.89 (-2281.61-1367.83)	0.624
$A\beta_{42}$	1.17 (-0.84-3.18)	0.254	-2.13 (-7.69-3.43)	0.452
AxD*$A\beta_{1-42}$	-0.42 (-1.36-0.53)	0.388	1.60 (-2.16-5.37)	0.404
	CC-G		**ILF L**	
	B (95%IC)	p[a]	B (95%IC)	p[a]
Model 1				
AxD	-855.54 (-1523.47--187.60)	**0.012**	-447.54 (-1515.27-620.18)	0.411
7K-C	-14.87 (-21.77--7.97)	**0.000**	2.84 (-6.72-12.41)	0.561
AxD *7K-C	9.61 (5.26-13.96)	**0.000**	-1.75 (-8.73-5.24)	0.624
Model 2				
AxD	-216.69 (-1753.82-1320.44)	0.782	1016.47 (-1347.23-3380.17)	0.399
$A\beta_{42}$	-1.28 (-5.60-3.05)	0.563	3.79 (-2.51-10.10)	0.238
AxD*$A\beta_{42}$	1.01 (-1.85-3.86)	0.490	-2.75 (-7.55-2.05)	0.261
	Hippoc. R			
	B (95%IC)	p[a]		
Model 1				
AxD	-45.98 (-413.78-321.81)	0.806		
7K-C	0.69 (-1.54-2.91)	0.546		
AxD *7K-C	-0.30 (-2.46-1.86)	0.785		
Model 2				
AxD	-19.19 (-962.30-923.92)	0.968		
$A\beta_{42}$	0.27 (-1.42-1.97)	0.750		
AxD*$A\beta_{42}$	-0.07 (-1.89-1.76)	0.942		

Note. Significant effects are shown in bold. B, beta coefficient; CI, confidence interval; $A\beta_{42}$ = β-amyloid$_{1-42}$; CC-S, corpus callosum-splenium; CC-G, corpus-callosum-genu; ILF L, inferior longitudinal fasciculus left; FRNX, fornix (column+body); Hippoc. R, hippocampus right, AxD, axial diffusivity; NfL, neurofilament light; 7K-C, 7-ketocholesterol.

b. Generalized linear models adjusted for gender, age, white matter hyperintensities and *APOE* genotype