Altered Circulating Free Oxysterols in Mild Cognitive Impairment: Relationship to Diet, Lipids and Genetics

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Abstract

Background

Oxysterols, products of cholesterol oxidation, seem dysregulated in Alzheimer’s disease (AD) and mild cognitive impairment (MCI). However, little is known about serum free oxysterol (biologically active form) profiles for MCI.

Methods

Serum free oxysterol profiles were measured by ultra-performance liquid chromatography-mass spectrometry in discovery (n = 145) and validation batch (n = 356), both of which were constituted with cognitively normal individuals and MCI patients. We assessed associations of oxysterols with demographics, cognition, diet, lipids and genetics.

Results

27-Hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholestenoic acid could distinguish MCI from control with good performance in both discovery batch (AUC = 0.80) and validation batch (AUC = 0.71). MCI status could affect correlation patterns among oxysterols, cognition and lipids. Genetic variants in APOE, CYP27A1, CYP46A1 showed inverse associations with cognition and lipids/oxysterol levels while CYP7A1 showed consistent associations.

Conclusions

We identify for the first time an altered free oxysterol profile and report associations with genetic variants in MCI using two-step case-control studies.

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1. Background

Dementia looms large worldwide as a major threat to public health when taking longevity continues to rise into consideration. Patients suffering from dementia worldwide in 2015 was estimated to be 46.8 million, which are believed to be predicted to 131.5 million by 2050[1]. As the most common dementia, Alzheimer’s disease (AD) accounts for 80% of cases. It is an irreversible neurodegenerative disease that comprises a broad range of clinical symptoms ranging from progressive cognitive decline to compromised activities of daily living (ADL) and histological features of extracellular deposits of amyloid β (Aβ) in senile plaques and intracellular hyperphosphorylation of tau protein in neurofibrillary tangles (NFT)[2]. Heretofore, AD has neither cures nor disease-modifying therapies. And it has been accepted that AD-related pathology occurs decades before the onset of clinical symptoms. Therefore, the focus of AD research is evolving to further encompass earlier stages in order to promote future approaches in
The mild cognitive impairment (MCI) denoted as a preclinical stage of AD with a subtle cognitive impairment with preserved function in ADL[4]. Biomarkers exploration for MCI will be a major advance for estimating the likelihood of possessing the underlying pathophysiology of AD, predicting the cognitive decline in the future and developing disease-modifying or even preventative therapies[5].

Oxysterols are oxidized derivatives of cholesterol or its sterol precursors with oxygen-containing modifications of hydroxyl, hydroperoxy, keto, epoxy or carboxyl moieties in the sterol ring and/or in the side-chain[6]. Generated either enzymatically by the mitochondrial cholesterol hydroxylases belonging to the group of cytochrome P450 family or non-enzymatic routes via endogenous free radical mediated oxidation as well as food rich in cholesterol during storing and cooking[7], oxysterols emerge as a large family of biologically active molecules involved in a plethora of physiological processes including synthesis of bile acids, lipid homeostasis, immune regulation, oxidative stress and inflammation[8]. Consequently, their levels in biological fluids and organs are greatly altered in specific pathologies and increasing evidence correlates pathological levels of oxysterols to the pathogenesis of various disease processes, including AD[9], which highlights their potential use as biomarkers in the diagnosis and treatment of diseases.

Increasing evidence is now consolidating the link between AD and oxysterols, which is related to oxidation-related neuropathologies and the link connecting dysregulated cholesterol homeostasis in the brain and hypercholesterolemia when taking the ability of oxysterols, unlike cholesterol, to permeate the blood brain barrier (BBB) into consideration[10]. In particular, the intra- and extracerebral balance of two side-chain enzymatical oxysterols, flux of 24-hydroxycholesterol from the brain into the circulation and the opposite flux of 27-hydroxycholesterol, has been identified as key aspects for AD pathogenesis supported by cerebral derangement associated with the disease as well as their involvement in modulating neuronal death, neuroinflammation, oxidative stress, Aβ accumulation and other molecular pathways underlying AD ethiopathology[11]. Besides, the oxysterols resulting from cholesterol autoxidation potentially implicated in AD pathogenesis is now emerging. By crossing the BBB, they can also flow from the brain into the circulation and vice versa and have been identified to be significantly increased during AD progression[12]. Therefore, oxysterol profiles, rather than single compound, in biological fluids and organs may be more useful as the biomarkers of diseases.

Current biomarker research for AD or MCI mainly focus on cerebrospinal fluid (CSF) profiles of oxysterols. A recent meta-analysis has analyzed published data and found that the CSF levels of cholesterol, 24-hydroxycholesterol and 27-hydroxycholesterol are significantly elevated in AD and MCI subjects compared to controls[13]. However, blood-based biomarkers could be a more attractive option due to reduced invasiveness, time- and cost-efficient and increased patient acceptance[14]. Unfortunately, the results of studies concerning about blood levels of oxysterols between AD/MCI patients and controls have been decidedly mixed. For example, although elevated 27-hydroxycholesterol may reflect neuropathological changes associated with MCI by our previous studies[15], some point toward the nonsignificant levels in (pre)demented patients[16]. An often neglected issue in the discussion of inconsistent oxysterol levels is oxysterols, like cholesterol itself, are present both as free form (biologically
active) and esterified form (largely biologically inert) in vivo[17]. It has been assumed and tested by Heverin et al[18] that it is the free oxysterol that can cross the BBB due to impermeability of lipoproteins that transport esterified oxysterols. The amount and the state of oxysterol esterification vary substantially[19]. Hence, separating free from esterified forms could provide more sensitive measure to identify blood-based biomarkers and disease-associated differences.

Genetic variability of catalyzing enzymes of oxysterols metabolism, e.g. cholesterol-24S-hydroxylase (CYP46A1), also has influence on neurodegenerative process[20]. A large bulk of literature points to associations between their genetic variability and AD incidence, progression, and therapy targets as well as the physiological role of genes[1]. Stiles et al[21] have demonstrated in 3230 participants by genome-wide study that serum oxysterol levels were strongly associated with genetic variation. Therefore, correlating oxysterol levels with genotype provides productive insight into human lipid metabolism and AD progression. The current research data about the role of genetic variability of oxysterol catalyzing enzymes in AD or MCI is still limited to CYP46A1 with low coverage of loci[22]. Other potential genetic loci for different enzymes still need clinical validation, which may allow the stratification of MCI patients into genetically defined groups for risk prediction and subsequent screening strategies.

The goal of the current liquid chromatography-mass spectrometry–based metabolomics research is to evaluate free oxysterol profiles for MCI and their associations with genetic variability and dietary and serum cholesterol. A total of 501 serum samples of participants from The Effects and Mechanism of Cholesterol and Oxysterol on Alzheimer’s disease (EMCOA) cohort in China were collected, and a two-step analysis strategy, including discovery and validation, was employed to identify and validate the clinical practicability of oxysterol profiles or specific species.

2. Methods

2.1 Study design and population

In this study, serum samples from 253 clinically diagnosed MCI patients and 248 cognitively healthy controls were collected for metabolomics measures and genotype. A two-stage case-control study design was performed for metabolomic analyses: an initial discovery batch (MCI = 75; Control = 70) to identify free oxysterol profiles for MCI diagnosis, which were further validated in the validation batch (MCI = 178; Control = 178). Both MCI cases and controls were from the same source population of the EMCOA study, a prospective, multicenter, observational cohort study of cognitive health in middle-aged and elderly Chinese aged 50–70 years[23]. Demographical information, medical history of chronic diseases, a broad range of neuropsychological tests and dietary surveys of all participants were collected. Informed consent was obtained from all enrolled participants. The medical Ethics Committee of Capital Medical University and the institutional review board at each study site approved the study (No. 2013SY35). The experimental flowchart of the current study is shown in Fig. 1.

2.2 Sample collection and quantification of free oxysterols
Targeted metabolomics profiling was performed to measure levels of 22 free oxysterols in serum samples. Morning fasting serum samples were collected, aliquoted and stored as described previously[24]. Oxysterols were extracted from serum, dried in the SpeedVac under OH mode and analyzed using an Exion UPLC system coupled with a triple quodrupole/ion trap mass spectrometer (QTRAP 6500 Plus, Sciex).

2.3 Single nucleotide polymorphism (SNP) selection and genotyping

Based on knowledge of metabolic pathways, transporters, potential targets and the mechanism of action of oxysterols, a total of 27 single nucleotide polymorphisms (SNPs) in 6 genes (CYP27A1, CYP46A1, CYP7A1, CYP7B1, CH25H and APOE) were selected as potential functional or frequently reported variants after extensive literature review and referring to several databases. Genomic DNA was extracted from peripheral blood using QIAamp DNA Blood Mini Kit (QIAGEN Valencia, CA, USA) in accordance with the manufacturer's protocols followed by being checked for quality and concentration. Sequencing was performed by the Sequenom MassARRAY iPLEX system or KASP genotyping assay as appropriate in BioMiao Biological Technology, Beijing, China. Criteria of SNP selection and detailed information about these SNPs are listed in Supplementary Table 1 and Table 2. The specific primers for each polymorphism are displayed in Supplementary Table 3.

2.4 Statistical analysis

Normality of continuous variables was assessed with Skewness–kurtosis normality test. Normally distributed variables are presented as the mean ± standard deviation and compared using two-tailed Student's t test for independent samples. Non-normally distributed variables are presented as the median (interquartile range) and compared with Wilcoxon rank sum test. The Pearson chi-squared test was used for categorical variables. Statistical differences between groups were considered significant if \( P < 0.05 \). Where indicated, the false discovery rate (FDR) with Benjamini–Hochberg method was used to correct \( P \) value for multiple testing. Receiver operating characteristic (ROC) curve analysis was performed and area under the curve (AUC) was calculated. Pearson or Spearman rank correlation coefficients were used to examine pairwise correlations. These analyses were conducted with R (version 3.5.1). With respect to genetic variant analysis, logistic regression analyses were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for allelic and genotypic associations with MCI risk adjusted by age or sex through PLINK (v1.07, USA) software. The Hardy–Weinberg equilibrium for each SNP and allele frequencies were analyzed with chi-squared test. Multiple linear regression was performed to assess the effects of genetic variants on cognitive performance, serum lipids and oxysterol levels in additive model. Statistical significance was defined as \( P \)-value < 0.05.

2.5 Data availability

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.
3. Results

3.1 General characteristics of the Discovery and Validation batches

The characteristics of discovery and validation batches are shown in Table 1. MCI cases and controls both in the discovery and validation batches showed comparable demographic characteristics, dietary intakes of cholesterol and fat as well as serum lipids (P > 0.05), demonstrating the general equivalence of the subjects used for the discovery and validation batches of the current analysis. However, global and multiple domain-specific cognitive measurements were significantly different between MCI and control groups (P < 0.05), as expected.
Table 1
General characteristics of the case-control subjects selected from the discovery and validation batches

|                          | Discovery batch | Validation batch |
|--------------------------|-----------------|------------------|
|                          | MCI (N = 75)    | Controls (N = 70) |         | MCI (N = 178) | Controls (N = 178) |         |
|                          | P value         |                  |         | P value       |                  |         |
| Demographic characteristics |                 |                  |         |               |                  |         |
| Women, n(%)              | 41 (54.7%)      | 33 (47.1%)       | 0.476  | 84 (47.2%)    | 80 (44.9%)        | 0.853  |
| BMI (kg/m2)              | 24.77 ± 3.10    | 24.27 ± 2.87     | 0.460  | 24.77 ± 2.94  | 24.06 ± 3.02      | 0.069  |
| Age                      | 58.61 ± 4.59    | 59.51 ± 3.74     | 0.354  | 60.02 ± 4.86  | 60.84 ± 4.45      | 0.641  |
| Education years          | 9.55 ± 2.55     | 10.10 ± 2.85     | 0.370  | 10.31 ± 2.61  | 10.49 ± 3.36      | 0.941  |
| Current smoker, n(%)     | 17 (22.7%)      | 12 (17.1%)       | 0.504  | 47 (26.4%)    | 38 (21.3%)        | 0.470  |
| Current drinker, n(%)    | 11 (14.7%)      | 15 (21.4%)       | 0.44   | 42 (23.6%)    | 56 (31.5%)        | 0.222  |
| Hypertension, n(%)       | 21 (28.0%)      | 26 (37.1%)       | 0.388  | 64 (36.0%)    | 60 (33.7%)        | 0.853  |
| Diabetes, n(%)           | 13 (17.3%)      | 6 (8.6%)         | 0.288  | 23 (12.9%)    | 20 (11.2%)        | 0.853  |
| CHD, n(%)                | 8 (10.7%)       | 5 (7.1%)         | 0.548  | 11 (6.2%)     | 10 (5.6%)         | 0.941  |
| Dietary intakes          |                 |                  |         |               |                  |         |
| Cholesterol, g/d         | 199.4 (131.1, 332.7) | 302.4 (120.5, 378.4) | 0.254  | 327.4 (167.8, 421.8) | 317.4 (173.4, 394.5) | 0.607  |
| Fat, g/d                 | 54.4 (44.6, 64.0) | 53.2 (43.1, 70.2) | 0.888  | 59.9 (45.9, 82.5) | 58.4 (47.8, 79.8) | 0.965  |
| SFA, g/d                 | 11.2 (9.2, 13.6) | 12.5 (8.7, 16.4) | 0.288  | 13.7 (9.9, 20.9) | 14.2 (10.3, 19.4) | 0.965  |
| PUFA, g/d                | 20.2 (15.1, 26.6) | 20.0 (14.1, 26.6) | 0.719  | 21.2 (15.2, 28.0) | 20.1 (16.3, 30.3) | 0.853  |
| MUFA, g/d                | 16.0 (12.5, 19.4) | 16.0 (12.3, 22.4) | 0.603  | 20.1 (13.8, 31.0) | 19.4 (14.1, 27.4) | 0.842  |
| Blood lipids             |                 |                  |         |               |                  |         |
| TC, mmol/L               | 4.46 (3.79, 5.07) | 4.38 (3.57, 4.83) | 0.476  | 4.69 (3.90, 5.34) | 4.51 (3.80, 5.22) | 0.397  |
| TG, mol/L                | 1.55 (1.04, 1.96) | 1.51 (1.08, 2.13) | 0.596  | 1.38 (1.0, 1.92) | 1.41 (1.03, 1.90) | 0.965  |
|                     | Discovery batch       | Validation batch       |      |
|---------------------|-----------------------|------------------------|------|
| HDL-C, mmol/L       | 1.2 (1.1, 1.4)        | 1.2 (1.0, 1.4)         | 0.637|
| LDL-C, mmol/L       | 2.6 (2.0, 3.2)        | 2.4 (2.0, 2.9)         | 0.298|
| Non-HDL, mmol/L     | 3.06 (2.56, 3.76)     | 2.99 (2.49, 3.57)      | 0.388|
| LDL-C/HDL-C         | 2.15 (1.67, 2.57)     | 1.87 (1.54, 2.51)      | 0.302|

**Cognitive Performance**

|                     | Discovery batch       | Validation batch       |      |
|---------------------|-----------------------|------------------------|------|
| MMSE                | 26 (24, 28)           | 29 (28, 30)            | < 0.001*|
| MoCA                | 21 (19, 23)           | 27 (25, 28)            | < 0.001*|
| AVLT-IR             | 13 (11, 16.50)        | 17 (12, 20)            | 0.021*|
| AVLT-SR             | 4 (2, 6)              | 6 (4, 7)               | 0.053 |
| AVLT-LR             | 3 (1, 5)              | 5 (3, 7)               | 0.021*|
| SDMT                | 31 (25, 36)           | 39 (30, 48)            | < 0.001*|
| LMT                 | 7.0 (4.5, 12.5)       | 10.5 (8.0, 14.5)       | 0.021*|
| TMT-A               | 70 (54, 92)           | 61 (49, 77)            | 0.101 |
| TMT-B               | 186 (147, 217)        | 140 (109, 180)         | < 0.001*|
| DSF                 | 7 (6, 8)              | 8 (7, 9)               | 0.032*|
| DSB                 | 4 (3, 4)              | 4 (4, 5)               | < 0.001*|
| SCWT                | 38 (31, 50)           | 37 (28, 44)            | 0.360 |

* indicates statistical significance.
### Abbreviations:

- MMSE: mini-mental state examination
- MoCA: Montreal Cognitive Assessment
- AVLT-IR: auditory verbal learning test-immediate recall
- AVLT-SR: auditory verbal learning test-short recall
- AVLT-LR: auditory verbal learning test-long recall
- SDMT: symbol digit modalities test
- LMT: logical memory test
- DSF: digit span forwards
- TMT-A: trail making test-A
- TMT-B: trail making test-B
- DSB: digit span backwards
- SCWT: Stroop color-word test
- BMI: body mass index
- CHD: coronary heart disease
- TC: total cholesterol
- HDL-C: high-density lipoprotein cholesterol
- LDL-C: low-density lipoprotein cholesterol
- TG: triglycerides
- Non-HDL-C: non-high-density lipoprotein cholesterol
- SFA: saturated fatty acid
- PUFA: polyunsaturated fatty
- MUFA: monounsaturated fatty

Data shown as mean ± standard deviation were compared between 2 groups using Student’s t test for independent samples;

Data shown as median (interquartile range) were compared between 2 groups using Wilcoxon rank sum test;

Data shown as n (%) were compared between 2 groups using the chi-square test;

*P* < 0.05 after false discovery rate adjustment

### 3.2 Serum free oxysterol profiles are significantly altered in MCI

We identified a total of 22 oxysterols by UPLC-MS. Of the detected oxysterols, 11 oxysterols which passed multiple testing correction of FDR were found to be significantly different between MCI cases and controls in the discovery batch, with all of the oxysterols having a higher concentration in case subjects (Supplementary Table 4, Fig. 2A). We then assessed the same oxysterols in the validation batch. However, in the validation batch, only 27-hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholestenoic acid surpassed the more stringent FDR corrected threshold for significance with a higher concentration in case subjects (Supplementary Table 4, Fig. 2B). Combining discovery and validation data, serum 27-hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholestenoic acid concentrations displayed a similar distribution (Fig. 2C&D), which indicated that serum 27-hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholestenoic acid are greatly increased in patients with MCI, being a potential diagnostic marker.

### 3.3 Diagnostic power for combination of differential oxysterols

Subsequently, ROC curve analyses were used to evaluate the ability of differential oxysterols to distinguish MCI patients from control subjects. ROC curve indicated that 27-hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholestenoic acid showed a promising diagnostic value with AUC 0.80 in the discovery batch (Fig. 3A). Similar diagnostic performance was observed in ROC curve analyses in the validation batch. Their levels also differentiated between MCI patients and controls with an AUC of 0.71 under the ROC curve, demonstrating good replication (Fig. 3B).
3.3 Correlation analysis

Correlations between all pairs of oxysterols demonstrated a majority of stronger positive correlations in general than the smaller number of negative correlations (Fig. 4A&B). We then examined the relationship of oxysterols with demographic characteristics, global and domain-specific cognitive performance, blood lipids as well as dietary cholesterol and fats in the discovery and validation batches of subjects (Fig. 4C&D). Some significant correlations of oxysterols with other variables were consistent in both two batches, such as 7α-hydroxy-4-cholesten-3-one with BMI, 7α-hydroxy-3-oxo-4-cholestenoic acid with MoCA and SDMT score, multiple oxysterols with blood lipids as well as 4α-hydroxycholesterol with dietary cholesterol. However, direction of some correlations was altered between discovery and validation batches, such as 25-hydroxycholesterol with age, 27-hydroxycholesterol with drinking and dietary cholesterol, 7-ketocholesterol with MoCA and 7α,25-dihydroxycholesterol with SDMT score.

We also examined the changes of consistent correlations in MCI cases and controls respectively in the chord diagrams. The correlation patterns among oxysterols, demographic characteristics, global and domain-specific cognitive performance, dietary cholesterol and fat as well as blood lipids differed markedly for cases and controls (Fig. 4E&F). In particular, some direction of intercorrelations of oxysterols was altered between cases and controls, suggesting that part of interconnectedness of oxysterols were interacting differently depending on cognitive status. Moreover, MCI subjects lost some correlations of oxysterols with blood lipids as well as intercorrelations of blood lipids but had more intercorrelations of cognitive performance. The loss of correlations in serum has major implications for how misalignment among cholesterol metabolism may be exacerbated by cognitive impairment.

3.4 Genetic association studies

The genotypic distribution of most SNPs in the control group of current study conformed to Hardy-Weinberg equilibrium (P > 0.05) except rs4145039 in CYP46A1 (P < 0.001, Supplementary Table 5), which was excluded from further analysis. The allele distribution, genotype frequencies of reserved SNPs in MCI cases and controls and their associations with MCI risk using logistic test including dominant, recessive and log-additive model were presented in Supplementary Table 6. One SNPs in APOE (rs7259620), 5 in CYP46A1 (rs8019753, rs8003602, rs7157609, rs4900442 and rs754203) and 1 in CYP7A1 (rs2081687) showed significant associations with MCI risk in univariate logistic regression analysis in different genetic models (Fig. 5A). These associations remained significant after adjustment for age and sex. However, another SNP in APOE (rs429358) only showed protective effects on MCI in dominant model (OR = 0.54; 95% CI, 0.29–0.99) and log-additive model (OR = 0.53; 95% CI, 0.29–0.96) after adjustment (Fig. 5B).

Linear regression with or without adjustment for age and sex was used to explore the association between genetic variants and cognitive performance, blood lipids and oxysterol levels. For cognitive performance, three SNPs (rs429358, rs7259620 and rs405509) located in APOE and combined genotype APOE4, two SNPs (rs6436087 and rs10713583) in CYP27A1, all of five SNPs in CYP46A1 and five SNPs in CYP7A1 (rs3808607, rs3824260, rs1023652, rs1457043 and rs2081687) showed significant impacts
on the cognitive tests for global cognition (MMSE and MoCA), verbal memory (AVLT-IR and AVLT-SR),
processing speed (SDMT, TMT-A and TMT-B), attention and working memory (LMT and DSF), indicating
that both global and domain-specific cognition may be influenced with the mutations of these alleles
(Supplementary Table 7). With respect to serum lipids and oxysterols (Supplementary Table 8&9), SNPs
in APOE, CYP27A1 and CYP46A1 associated with better cognition was inversely associated with TC, HDL-
C, LDL-C and multiple oxysterol levels while SNPs in CYP7A1 associated with better cognition was
positively associated with TC, Non-HDL-C and multiple oxysterol levels; SNPs in CYP46A1 associated
with poorer cognition was positively associated with LDL-C level while SNPs in CYP7A1 associated with
poorer cognition was inversely associated with TC, LDL-C, Non-HDL-C and multiple oxysterol levels. In
addition, SNPs in CYP7B1 (rs16931331, rs16931334 and rs7829301) and CH25H (rs4078488 and
rs17117126) not associated with cognition still had significant impact on serum lipids and oxysterol
levels. The Sankey diagrams were plotted to demonstration significant associations of SNPs with
cognitive tests, serum lipids and oxysterols (Fig. 5C&D).

4. Discussion

In this study, we used the ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) to
quantify 22 free oxysterols in serum in two groups of subjects from the EMCOA study and establish that
serum free oxysterol profiles are significantly altered in MCI patients. Using ROC analyses, we identified
27-hydroxycholesterol along with its brain metabolite, 7α-hydroxy-3-oxo-4-cholestenoic acid, could
distinguish MCI patients from control subjects with good performance in both the discovery batch (AUC =
0.80) and the validation batch (AUC = 0.71). We then correlated oxysterols with demographics, cognition,
dietary cholesterol and fats, serum lipids and genotype and uncover how variation in specific oxysterols
are linked with interindividual disparities in diet, lipids, cognition and genetic variation and how these
connections are modified by cognitive status.

Accumulating research has reported significant changes in the brain concentrations and CSF levels of
several oxysterols in AD or MCI patients[25, 26], which may be triggered initially by pathological changes
of oxysterols in peripheral circulation. However, the measurement of blood oxysterols in MCI patients
previously has gained considerable controversy and revealed little consistency[27−29]. Therefore, several
unique features were incorporated into the current study in an attempt to overcome limitations of prior
work and derive a more comprehensive and accurate understanding of deranged oxysterol profiles in
MCI. First, rather than focusing on limited species of oxysterols, we evaluated a broader range of 22
oxysterols and their secondary metabolites. Second, the lack of verification or validation of candidate
oxysterol biomarkers have been the bottlenecks of earlier research. To make the results more convincing,
two independent data sets from EMCOA study were included and the initial finding in the discovery batch
was further validated by a larger number of MCI patients and controls in the validation set, guaranteeing
the reproducibility of our results. Third, saponification technique has been widely used in the previous
research to extract “total” oxysterols without discriminating between free (biologically active) and
esterified forms (largely biologically inert)[17]. In line with several most recent reports[30−32], the current
study focuses on the free rather than esterified oxysterols with more sensitive method for separation and
quantitative determination when taking the reasonable assumption that it is the free oxysterols that is crossing the BBB into consideration.

Here we present the discovery and validation of serum free oxysterol changes that distinguish cognitively normal participants from MCI patients. The defined two-oxysterol penal features the most common side chain oxysterol of 27-hydroxycholesterol, which mainly derives from the peripheral circulation and flows into the brain[33], and its brain-derived end metabolite, 7α-hydroxy-3-oxo-4-cholestenoic acid, to which 27-hydroxycholesterol is converted in the brain through enzymes of CYP27A1, CYP7B1 and HSD3B7 catalyzed oxidation, is then eliminated in the systemic circulation and in the CSF[12]. We also observed consistent and strong negative correlations between 7α-hydroxy-3-oxo-4-cholestenoic acid and global cognition, indicated by MoCA score, as well as processing speed performance, indicated by SDMT score. Saeed et al[34] and Crick et al[31] have demonstrated that the concentration of 7α-hydroxy-3-oxo-4-cholestenoic acid was remarkably elevated in CSF both from patients with a dysfunctional BBB and neurodegenerative disease including AD and Parkinson’s disease (PD), or amyotrophic lateral sclerosis (ALS), which may be likely to reflect increased brain accumulation of 27-hydroxycholesterol as a result of damaged BBB integrity and increased serum levels of 27-hydroxycholesterol in MCI patients. The key role of 27-hydroxycholesterol in AD pathogenesis has been strongly supported by our previous animal and cellular research, pointing to the involvement of 27-hydroxycholesterol in the pro-inflammatory molecule release[35], oxidative stress[36], lysosomal membrane permeabilization and pyroptosis[37], synaptic dysfunction[38] and Aβ production[39]. We thus discovered and validated serum 27-hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholestenoic acid as diagnostic biomarkers in MCI. It is noteworthy that the currently presented serum biomarker panel of free oxysterols is especially for patients with early stages of AD development. Some studies have pointed that the brain conversion of 27-hydroxycholesterol into 7α-hydroxy-3-oxo-4-cholestenoic acid may be regarded as a protective mechanism since 7β-hydroxylated intermediates of the conversion, catalyzed by the neuronal enzyme CYP7B1, seem to be less cytotoxic[18]. The markedly reduced expression of CYP7B1 arises in the late stage of AD due to severe neuron loss and then leads to producing little or no amounts of 7α-hydroxy-3-oxo-4-cholestenoic acid[40]. Therefore, the application of this biomarker panel may be challenging in patients with long disease duration.

The relatively modest increase of other circulating oxysterol concentrations only in discovery batch suggests that these oxysterols may have limited utility as disease markers in MCI. However, many of them, especially nonenzymatical oxysterols deriving from cholesterol autoxidation, have been reported to be associated with AD progression due to their ability to stimulate inflammation[25]. Besides, if there is an elevated flux of 27-hydroxycholesterol into the brain as a consequence of a disturbed BBB, there may also be an increased flux of other oxysterols. Therefore, it may be assumed that in MCI or “compensated” AD stages, serum oxysterol levels are initially unchanged or slightly increased despite of presence of pro-oxidant and inflammatory stressors. However, in the “de-compensated” AD state, oxysterol homeostasis is overwhelmed by failure of cholesterol homeostatic mechanisms and widespread neuronal degeneration. These results highlight the importance of elaborating oxysterol profile changes among the different stages of AD so as to prevent or decrease neuronal damage.
The study taken here identified numerous relationships of serum levels of oxysterols with blood lipids, dietary cholesterol and fats and demographics. Common origins from cholesterol may explain many positive correlations with blood lipids. For example, the origin related to formation by enzymatic pathways may explain the consistent positive associations of 4β-hydroxycholesterol and 24-hydroxycholesterol with TC in both two batches. Besides, the consistent strong positive associations of 24-hydroxycholesterol with both HDL-C and LDL-C may also underlie the previously reported[41] cotransport of 24-hydroxycholesterol mainly with lipoprotein particles of HDL and LDL in the circulation. In addition to endogenous sources, oxysterols may also derive from the diet with food containing cholesterol and animal fat especially during food storing and cooking[42]. Findings in the validation batch including subjects with relatively higher dietary cholesterol and fats intakes demonstrated most positive correlations of oxysterols with dietary variables. Unexpectedly, consistent negative correlations of 4α-hydroxycholesterol and inverse correlations of 27-hydroxycholesterol with dietary cholesterol were observed, suggesting the dietary sources may be overwhelmed by endogenous sources. Taking into account that oxysterols can be further metabolized to bile acids, they play an important role of balance between cholesterol absorption and excretion[43].

A majority of positive correlations between all pairs of oxysterols were identified in the discovery batch, where smaller number of negative correlations were also presented. Shared metabolic pathways and precursor-product relationships may explain stronger positive correlations. However, the validation batch with increased MCI cases demonstrated more negative correlations between oxysterols. Consequently, we report emerging negatives correlations between oxysterols in MCI patients and the results suggested that the changes of interplay between oxysterols may be important indicators of cognitive status.

Genetic association analyses revealed significant associations between variants in genes encoding proteins or enzymes that are known to transport, synthesize and metabolize the oxysterols and cognition, blood lipids and oxysterol levels. Previous genetic association studies in AD or MCI have robustly identified several genetic risk variants in APOE and CYP46A1[44, 45], the latter of which specifies sterol 24-hydroxylase in the brain. In our analyses, five genetic variants in APOE, CYP46A1 and CYP7A1 were associated with decreased MCI risk while another three variants in CYP46A1 with increased MCI risk in specific genotype models, highlighting the heterogeneous effects of different loci in CYP46A1 for MCI development. We also investigated the association of blood lipids and serum oxysterol profiles with genetic variants in six oxysterol-related genes. On one hand, the inverse associations of rs6436087 in CYP27A1 with MMSE score and 7α-hydroxy-3-oxo-4-cholestenoic acid levels suggested that CYP27A1 may influence the risk of MCI through 27-hydroxycholesterol metabolism or changes in the brain. On the other hand, the consistent associations of rs3808607 in CYP7A1 with DSF score and 7α-hydroxy-4-cholesten-3-one indicated that CYP7A1 enzyme that catalyzes an essential step in bile acid synthesis[46] may influence MCI risk through cholesterol excretion. It is noteworthy that the seemingly protective role of genetic variants in APOE contrary to previous consensus[47], especially APOE4, may be due to significantly lower distribution of risk alleles of APOE ε4 in our population. These results should be interpreted with caution owing to ethnic homogeneity[48].
A limitation is the cross-sectional nature of the case-control studies, and therefore we can only detect correlations between clinical phenotypes and oxysterol levels but not draw causal conclusions. Longitudinal design would be better for in-depth examinations of dynamic changes of oxysterol biomarkers and predictive ability for the progression from prodromal to probable AD. Another limitation is lack of comparison with AD patients, thereby limiting the application of oxysterol biomarkers for discriminating among the different stages of AD, which may be more helpful to clarify dynamic changes of oxysterols and develop precise and targeted preventive and therapeutic strategies at the appropriate time.

5. Conclusions

In summary, we discovered and validated MCI associated serum free oxysterol profiles, the capacity of a biomarker panel to diagnose MCI patients and correlations with diet, lipids, cognition and genetics, underscoring the importance of oxysterol metabolism in the early stage of AD. The availability of a well-phenotyped Chinese population cohort enables informative explorations to be derived from static measurements of oxysterols. Additional studies may allow the investigation of mechanisms underlying observed correlations and to identify whether oxysterol levels are preventively and therapeutically significant.

Abbreviations

AD: Alzheimer’s disease; MCI: mild cognitive impairment; BBB: blood brain barrier; MMSE: mini-mental state examination; MoCA: Montreal Cognitive Assessment; AVLT-IR: auditory verbal learning test-immediate recall; AVLT-SR: auditory verbal learning test-short recall; AVLT-LR: auditory verbal learning test-long recall; SDMT: symbol digit modalities test; LMT: logical memory test; DSF: digit span forwards; TMT-A: trail making test-A; TMT-B: trail making test-B; DSB: digit span backwards; SCWT: Stroop color-word test; BMI: body mass index; SCWT: Stroop color-word test; CHD: coronary heart disease; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; Non-HDL-C: non-high-density lipoprotein cholesterol; SFA: saturated fatty acid; PUFA: polyunsaturated fatty; MUFA: monounsaturated fatty acid.

Declarations

Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.
Consent for publication

All the co-authors and participants have given their consent for publication in Molecular Neurodegeneration.

Ethics approval and consent to participate

The study design was ethically approved by the Ethics Committee of Capital Medical University (2013SY35). All participants were provided written informed consent at the beginning of the study.

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Author Contribution

RX conceived and designed the study, YA performed the analyses and wrote the manuscript. HY, XZ, YW, WL, TW and LW helped collect and analyze the data. All authors read and approved the final manuscript.

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Figures
Figure 1

Study flow chart
Figure 2

The volcano plots of 22 oxysterol levels between MCI and control groups in the discovery batch (A) and validation batch (B). 11 exhibited significant differential abundance (false discovery rate corrected P value <0.05) in the discovery batch with 7 of them presenting absolute fold change > 2 (larger red circle) and 4 of them presenting absolute fold change > 1 (smaller pink circle) when comparing MCI patients to control. Only 2 still exhibited significant differential abundance in the validation batch (false discovery rate corrected P value <0.05). Mann-Whitney U tests were used to calculate statistical significance. Differentially abundant metabolites with different fold change were individually color-coded. Serum levels of 27-droxycholesterol (μM) in MCI patients and control in two batches (C). Serum levels of 7α-hydroxy-3-oxo-4-cholestenoic acid (μM) in MCI patients and control in two batches (D). **P<0.01 after false discovery rate correction.
Figure 3

ROC curve analyses for diagnostic potential of 27-hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholestenoic acid in discovery batch (A) and validation batch (B).
Correlations between all pairs of oxysterols were presented by the dot plot in discovery batch (A) and validation batch (B). A bipolar gradient between red (positive correlation) and blue (negative correlation) is used in discovery batch and red (positive correlation) and green (negative correlation) used in validation batch. Circle size indicates FDR-corrected statistical significance. Heat maps display correlations of oxysterols with demographics, cognition, diets and blood lipids in the discovery batch (C) and validation batch (D). A bipolar color progression as indicated by the scale on the right of the figure between red (positive correlation) and blue (negative correlation) is used for discovery batch and red
(positive correlation) and green (negative correlation) for validation batch. Asterisks indicate FDR-corrected statistical significance. *P < 0.05; **P < 0.01. Chord diagrams showed the consist and significant (FDR corrected P<0.05) correlations for both batches in MCI patients (E) and control subjects (F) respectively. The oxysterols, cognition, demographics, diets and blood lipids are represented by colored sectors around the outside of the circle. Sectors are linked if significant correlations have been obtained between every pair of variables. Red indicates positive correlation and green indicates negative correlation. Band width corresponds to number of correlated pairs.

Figure 5

Forest plots present significant associations of SNPs with MCI risk using logistic regression including dominant, recessive and log-additive models without (A) or with adjustment of sex and age (B). Red indicates associations with increased MCI risk and green indicates associations with decreased MCI risk. Graphical summary by Sankey diagrams presented significant associations between cognitive performance and genes (C) as well as between blood analytes and genes (D). Abbreviations: MMSE: mini-mental state examination; MoCA: Montreal Cognitive Assessment; AVLT-IR: auditory verbal learning
test-immediate recall; AVLT-SR: auditory verbal learning test-short recall; AVLT-LR: auditory verbal learning test-long recall; SDMT: symbol digit modalities test; LMT: logical memory test; DSF: digit span forwards; TMT-A: trail making test-A; TMT-B: trail making test-B; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; Non-HDL-C: non-high-density lipoprotein cholesterol.

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