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Research paper

Effects silymarin and rosuvastatin on amyloid-carriers level in dyslipidemic Alzheimer's patients: A double-blind placebo-controlled randomized clinical trial

Auob Rustamzadeh [a,g], Nader Sadigh [b], Zahra Vahabi [c], Fatemeh Khamseh [d], Nafiseh Mohebi [e], Zahra Ghobadi [f], Fatemeh Moradi [a,*]

[a] Department of Anatomy, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[b] Department of Emergency Medicine, School of Medicine, Trauma and Injury Research Center, Iran University of Medical Sciences, Tehran, Iran
[c] Department of Geriatric Medicine, Ziaeian Hospital, Tehran University of Medical Sciences, Tehran, Iran
[d] Department of Neurology, Faculty of Medicine, Islamic Azad University, Tehran, Iran
[e] Department of Neurology, Rasool Akram Hospital, School of Medicine, Iran University of Medial Sciences, Tehran, Iran
[f] Neuroimaging and Clinical Biomarkers Research Group, Pars Darman Medical Imaging Center, Karaj, Iran
[g] Department of Anatomical Sciences, Faculty of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran

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ABSTRACT

Purpose: The production/excretion rate of Amyloid-β (Aβ) is the basis of the plaque burden in alzheimer's disease (AD), which depends on both central and peripheral clearance. In this study, the effect of silymarin and rosuvastatin on serum markers and clinical outcomes in dyslipidemic AD patients was investigated.

Methods: Participants (n=36) were randomized to silymarin (140 mg), placebo, and rosuvastatin 10 mg orally three times a day for 6 months. Serum collection and clinical outcome tests were performed at baseline and after completion of treatment. Lipid profile markers, oxidative stress markers, $A\beta_{1-42}/A\beta_{1-40}$ ratio, and Soluble Low-density lipoprotein receptor-Related Protein-1 (sLRP1)/Soluble Receptor for Advanced Glycation End Products (sRAGE) ratio were measured.

Results: There was a statistically significant increase in Δ-high density lipoprotein (ΔHDL) between silymarin and placebo ($P<0.000$) and also between rosuvastatin and placebo ($p=0.044$). The level of Δ-triglycerides (ΔTG) in the silymarin group has a significant decrease compared to both the placebo and the rosuvastatin group ($p<0.000$ and $p=0.036$, respectively). The Δ-superoxide dismutase (ΔSOD) level in the silymarin group compared to placebo and rosuvastatin had a significant increase ($p<0.000$ and $p=0.008$, respectively). The $\Delta A\beta_{1-42}/A\beta_{1-40}$ in the silymarin group compared to both the placebo and rosuvastatin groups had a significant increase ($p<0.05$). There was an inverse relationship between ΔTG and $\Delta A\beta_{1-42}/A\beta_{1-40}$ ($p=-0.493$ and $p=0.004$). $\Delta A\beta_{1-42}/A\beta_{1-40}$ has a direct statistical relationship with ΔSOD marker ($p=0.388$ and $p=0.031$). Also, there was a direct correlation between the level of $\Delta A\beta_{1-42}/A\beta_{1-40}$ and ΔsLRP1/sRAGE ($p=0.491$ and $p=0.005$).

Conclusion: Our study showed the relationship between plasma lipids, especially ΔTG and ΔHDL, with $\Delta A\beta_{1-42}/A\beta_{1-40}$ in dyslipidemic AD patients, and modulation of these lipid factors can be used to monitor the response to treatments.

1. Background

Alzheimer's disease (AD) is a neurodegenerative disorder involving the central nervous system (CNS) with a prominent symptom of progressive decline in cognitive function (Waldemar et al., 2007). AD pathology is mostly illustrated by extracellular accumulations of amyloid-beta (Aβ) peptides in senile plaques and neurofibrillary tangles as a result of intracellular deposits of hyperphosphorylated tau (hp-tau) protein (Long and Holtzman, 2019; Mohammadi et al., 2024). In late-onset AD (LOAD) the disease progressed with the symptom onset of over 60 years of age, whereas early-onset AD (EOAD) appears earlier approximately between 30 and 60 years of age (Bali et al., 2012). $A\beta_{1-42}$

* Correspondence to: Department of Anatomy, School of Medicine, Iran University of Medical Sciences, Tehran Postal code: 1449614535, Iran.
E-mail address: F7moradi@gmail.com (F. Moradi).

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deposition in the brain is the prominent sign of the late-onset AD (LOAD) (Calabrò et al., 2021). Nearly 50 million people worldwide are living with dementia and it is predicted to reach about 152 million people in 2050. This fact imposes an additional socio-economic and cultural burden on patients, families, and health organizations (Selkoe and Hardy, 2016; Wu et al., 2017). Despite the physiological character of $A\beta_{1-42}$ production, an imbalance in the $A\beta_{1-42}$ production/excretion rate is the basis of its increased level in AD. $A\beta_{1-42}$ accumulation is the causative factor of AD leading to synaptic loss, hp-tau protein, inflammation, oxidative stress (OS), and apoptosis (Sanabria-Castro et al., 2017). One of the major characteristics of LOAD is the comprehensive defect in peripheral $A\beta_{1-42}$ clearance (Mawuenyega et al., 2010) that is carried out independently from neurons and microglia (Zolezzi et al., 2014). The distribution and transfer of this physiologic process in the peripheral blood accounts for 50 % of the total brain $A\beta_{1-42}$ clearance (central clearance) in humans, indicating that the physiological clearance of $A\beta_{1-42}$ from peripheral organs is an important mechanism to prevent the accumulation of this peptide in the brain (Roberts et al., 2014). In blood, Low density lipoprotein receptor-related protein 1 (LRP1) is the key factor in peripheral $A\beta_{1-42}$ transport (Sagare et al., 2012). In contrast, the RAGE is the main receptor responsible for Aβ transfer from blood to brain parenchyma that in AD increases (Ullah and Lee, 2023). Increasing the binding affinity of $A\beta_{1-42}$ to LRP1 accelerates its clearance, which illustrates that this binding prevents $A\beta_{1-42}$ re-entry into the brain (Sagare et al., 2013). Approximately 70 % of $A\beta_{1-42}$ in plasma is directly bound to LRP1 forming an important part of the endogenous "sink effect apparatus" whose main function is to clear peripheral $A\beta_{1-42}$. LRP reduces the amount of free $A\beta_{1-42}$ in circulation and enhances the clearance mediated by cell-surface LRP across the blood-brain barrier (BBB) to the outside of the brain (Sagare et al., 2007). In AD patients, despite the reduction in expression of LRP1 in the vessels and total brain parenchyma (Osgood et al., 2017) it was found to be upregulated in neurons and active astrocytes around senile plaques (Arélin et al., 2002). Dyslipidemia can induce signaling cascades resulting in neuroinflammation, neurodegeneration, oxidative stress, hp-tau, and $A\beta_{1-42}$ accumulation as the key characteristics of AD pathology (Wanamaker et al., 2015). An increase in cholesterol concentration remarkably leads to an increase in the production of $A\beta_{1-42}$ resulting in amyloid deposits and neurotoxicity (Wolozin, 2002). Memory impairment as a consequence of hypercholesterolemia has been reported to be associated with loss of dendritic integrity, cholinergic dysfunction, inflammation, increased $A\beta_{1-42}$, and hp-tau in the cerebral cortex (Granholm et al., 2008; Ullrich et al., 2010). In the study of Murata et al. (2010), AD mice that received a 0.1 % silymarin diet for 6 months, the Aβ oligomerization and deposition in the brain were reduced and improvement in behavioral disorders was observed (Murata et al., 2010). Guo et al. (2019) revealed that silymarin can reduce the production of Aβ by inhibiting the β-amyloid precursor protein and can inhibit the Aβ polymerization into fibrils. Silymarin can not only increase the content of acetylcholine in the nervous system by inhibiting the activity of cholinesterase but also has antioxidant effects and modulates inflammatory responses (Guo et al., 2019). The administration of 200 mg/kg silibinin causes the conversion of $A\beta_{1-42}$ and acetylcholinesterase (AChE) into stable complexes in vitro, reduction of the $A\beta_{1-42}$ accumulation as well as mitigating the AChE activity. Since silymarin can cross the BBB and acts as a dual inhibitor of $A\beta_{1-42}$ peptide aggregation and AChE, it is proposed as a potential therapeutic approach in AD (Duan et al., 2015). Furthermore, long-term treatment with statins can alleviate AD symptoms, suggesting that alterations in lipid metabolism have a key role in its pathogenesis (Shepardson et al., 2011a, 2011b). Neuroinflammation is a trigger of the pathophysiological signaling in AD, in which NF-κB and reactive oxygen species (ROS) are actively involved (Husain et al., 2017). Molecular docking studies demonstrated that rosuvastatin has a high affinity for binding to proteins such as NF-κB, AChE, and $A\beta_{1-42}$ (Husain et al., 2017, 2018). A recent study illustrated that the risk of AD is reduced by 28.1 % in patients taking statins (Chu et al., 2018). Considering the rising number of AD patients in the world and the demand to investigate the effectiveness of novel medications, the present study was conducted to investigate the effect of silymarin (compared to rosuvastatin) on blood markers and cognitive status of mild AD patients with secondary dyslipidemia.

2. Patients and methods

2.1. Participants and eligibility criteria

A total number of 36 participants were enrolled in this study from AD patients referred to the neurology and geriatric center of Roozbeh Psychiatry Hospital, Rasoul Akram Hospital, Ziaeian Hospital, Jam`s Memory Clinic, and Noor Neurology Clinic in Tehran, between October 2021 and March 2022. Standard neuropsychological tests, including Montreal Cognitive Assessment (MoCA) or Mini-mental state examination (MMSE), was taken from all participants. In addition, patients take a blood sample to evaluate their lipid profiles, including triglyceride (TG), low-density lipoprotein (LDL), total cholesterol (TC), and high-density lipoprotein (HDL), to assess whether there is a sign of dyslipidemia to be included in the study. The list of inclusion criteria is as follows: 1-Confirmation of mild sporadic Alzheimer's disease based on neurologist's diagnosis and neuropsychological clinical questionnaire, including MoCA and MMSE with a total score of 19–24; 2-Justification of secondary dyslipidemia based on biochemical tests and demographic questionnaire; and 3-Confirmation of routine MRI imaging based on atrophy of the hippocampus and enlargement of cerebral ventricles. The following conditions were specified as exclusions in the study: no brain tumors; no active rheumatological disorders; no active epilepsy; diabetes; uncontrolled hypertension; no brain surgery; heart failure; chronic kidney failure; thyroid disorders; and no use of alcohol, opioid drugs, or cigarettes. Using additional therapeutic interventions, failing to follow medication instructions in the protocol, claustrophobia during magnetic resonance imaging (MRI), COVID-19 infection, and traumatic injury sustained during the study are among the exclusion criteria.

2.2. Grouping, intervention, and allocation

This study was implemented through block randomization and the patients was placed in the following three groups containing 12 patients (Fig. 1):

1- The silymarin group: The first intervention group: In addition to taking routine medications (donepezil or Rivastigmine), patients received 140 mg silymarin tablets (Livergol 140 mg manufactured by GolDaro Pharmaceutical Company; Isfahan, Iran) three times a day for 6 months.

2- The placebo group: In addition to taking routine medications (Donepezil or Rivastigmine), patients received a 140 mg placebo tablet (manufactured by GolDaro Pharmaceutical Company; Isfahan, Iran) three times a day for 6 months. From the perspective of blinding the study, placebo tablets were used for silymarin but the treatment with rosuvastatin was open-label.

3- The rosuvastatin group: In addition to taking routine medications (donepezil or Rivastigmine), patients received 10 mg of rosuvastatin (Ropixon, Abidi Pharmaceutical Company; Tehran, Iran) three times a day for 6 months.

The questionnaire list of demographic information of the patients was completed with the aid of the patient's companion. Through the interview, information, including education level, marital status, brand and number of coronavirus vaccine injected, occupation, blood glucose level, history of cardiovascular diseases, smoking, list of prescribed medication, and family history of obesity were obtained. It should be noted that the accuracy of this information was reconfirmed by evaluating their medical records. Moreover, after the medication prescription, at the end of the intervention, the participants were reinterviewed to fill in their demographic information along with the serious adverse event

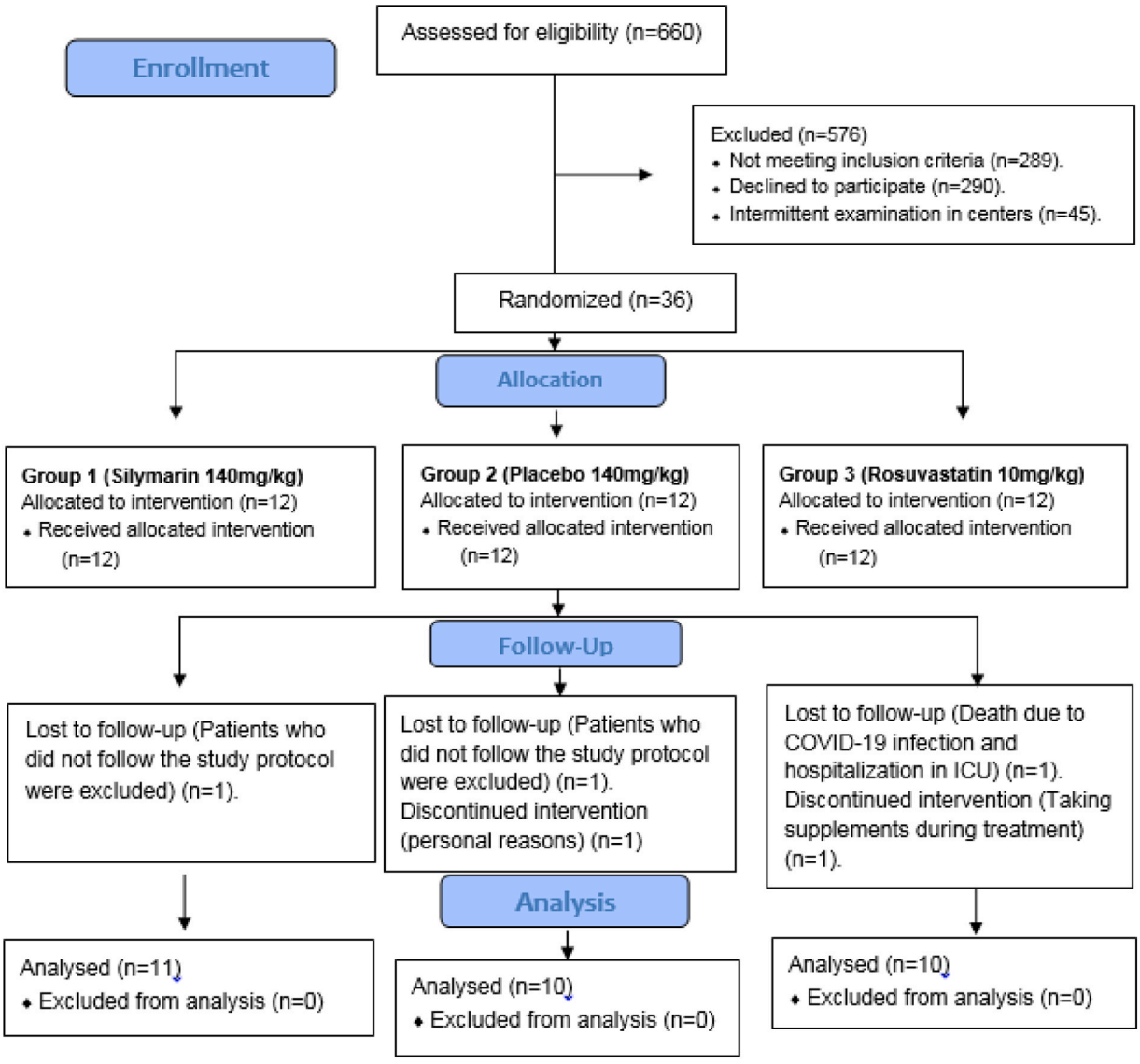

Fig. 1. Study flowdiagram according to international CONSORT guidelines.

(SAE) related to the assigned treatment.

2.3. Clinical outcome tests

All patients were included in the study based on medical evidence and the diagnostic decision made by the neurologist by considering the guidelines of the International Alzheimer's Institute (Neugroschl and Wang, 2011): a) the presence of a new complaint of the individual's memory, which should preferably be confirmed by a normal person, b) objective evidence in short-term memory disorder, c) defects in general cognitive function, and d) poor cooperation in performing usual social activities or other activities of daily life (Sperling et al., 2011). Two neuropsychological tools were used in the clinical evaluation of AD, including MMSE and Clinical Dementia Rating (CDR).

2.4. Blood sampling and measuring lipid profile

From each patient, 6 mL of venous blood was taken before and after medication intervention, and transferred to tubes with clot-activating gel (VacuLab® SSGT model, Liuyang SANLI Medical Technology Development Co., China). Subsequently, the tubes were centrifuged at 4000 rpm for 8 minutes. Soon after they were aliquoted in a microtube under the hood and in a clean room to prepare to be transferred to the −80 °C freezer (Gorgich et al., 2024). The levels of HDL, TG and TC, and liver enzymes, including aspartate transaminase (AST) and Alanine transaminase (ALT) were measured using the lipid kits (Delta Darman Part Co,; Tehran, Iran) based on enzymatic principles using an auto-analyzer (model Pictus 700, company Diatron). Furthermore, the level of LDL cholesterol was calculated using the Friedewald equation:

$$LDL = TC - HDL - 0.2 \times TG$$

2.5. Malondialdehyde (MDA) measurement

The MDA/thiobarbituric acid reactive substance (TBARS) assay kit (ZellBio GmbH, Hamburg, Germany) is a simple, reliable, and standardized tool for the assessment of lipid peroxidation in serum. The basis of the MDA test uses the MDA-TBA combination, which consists of the reaction of MDA and thiobarbituric acid (TBA) at high temperatures (Heidari et al., 2021). Malondialdehyde is measured colorimetrically at 535 nm in an acidic environment and at a temperature of 90 °C. The sensitivity of the kit for MDA measurement was 0.1 μM.

2.6. Measurement of superoxide dismutase (SOD)

The SOD assay kit (ZellBio GmbH, Hamburg, Germany) uses superoxide anion to convert to hydrogen peroxide and oxygen under enzymatic reaction for the quantitative measurement of samples. Afterward, the chromogenic product is measured by the colorimetric method (calorimetry) at 420 nm. The sensitivity of the ZellBio kit for SOD level was 1 U/mL.

2.7. Measurement of catalase activity (CAT)

The CAT kit (ZellBio GmbH, Hamburg, Germany) is a simple and standardized tool to evaluate lipid peroxidation in serum samples by calorimetric method at 405 nm. In this method, hydrogen peroxide is decomposed into water and oxygen by catalase enzyme. A unit of enzyme activity is the amount of enzyme that causes the production of one millimol of H_2O_2 per minute at an initial concentration of 10 mmol/liter at pH=7.4 and 37 °C temperature. The sensitivity of the kit for measuring SOD in serum was equal to 0.5 U/mL.

2.8. Measurement of $A\beta_{1-42}/A\beta_{1-40}$ peptide serum level

The kit used in the study was an enzyme-linked immunosorbent assay (ELISA) of double biotin antibody sandwich type for the quantitative measurement of $A\beta_{1-42}$ and $A\beta_{1-40}$ (Human $A\beta_{1-42}$ and $A\beta_{1-40}$ peptide ELISA Kit- ZellBio GmbH, Hamburg, Germany). The assay is based on adding the $A\beta_{1-42}$ protein to the wells that were already coated with the $A\beta_{1-42}$ monoclonal antibody. $A\beta_{1-42}$ antibodies labeled with biotin were added, which caused a streptavidin-HRP combination and the formation of an immune complex. The unbound enzymes after incubation and washing were removed. After adding substrate, A and B, the solution first turned blue and turned yellow under the influence of acid. The absorbance (OD) of each well was measured at a wavelength of 450 nm. According to the concentration of the standards and the corresponding OD values, the standard curve was drawn using the point-by-point calculation mode. The sensitivity of this kit was 1 ng/L to measure the serum level of the $A\beta_{1-42}$ peptide. These steps were also performed for $A\beta_{1-40}$ peptide according to the relevant kit.

2.9. Measurement of LRP/sRAGE peptide

In this study, the double biotin antibody sandwich ELISA method was used for quantitative measurement of LRP1 peptide and the sensitivity of the kit (Human LRP1 ELISA kit- ZellBio GmbH, Hamburg, Germany) was 0.09 mg/L. The frozen serum samples were transmitted from −80°C to 6°C place until the samples reached their melting point. Subsequently, they were kept at room temperature for 10 minutes to be prepared for the assessment. Before starting the experiment, all the solutions were transferred from the temperature of 4°C to room temperature. Then the necessary materials and buffers were prepared according to the instructions mentioned in the kit immediately before the start of the experiment. The intensity of the color produced in the wells was measured at a wavelength of 450 nm. The final concentration was calculated by the standard curve obtained using the absorbance obtained from different defined dilutions. sRAGE measured based on a sandwich ELISA kit (Human sRAGE ELISA kit- ZellBio GmbH, Hamburg, Germany). The samples were centrifuged at 1000xg for 20 minutes. The detection antibody was the biotin-conjugated anti-sRAGE antibody. Then, the pilot samples and standards were added to the wells. The coated antibody was then exposed to a biotinylated detection antibody, which bound to the sRAGE conjugated on it. HRP-Streptavidin was added after unbound conjugates were washed off. TMB substrates were added to observe the HRP enzymatic reaction following a third washing. TMB was catalyzed by HRP to produce a blue product, which changed to yellow upon the addition of a stop solution. After that, a standard curve was created by reading the O.D. absorbance in a microplate reader at 450 nm.

2.10. Data analysis

Statistical analyses were performed using SPSS software (V26; IBM Corp., Armonk, NY, United States). Therefore, to facilitate data analysis codes A, B, and C were given to the statistics consultant by the clinical supervisor. The value of each variable corresponding to the before and after the intervention was subtracted from each other to obtain the difference (Delta- Δ) for each variable. Moreover, mean±standard deviation was utilized for the descriptive report of quantitative values. In addition, qualitative data were reported as a frequency in percentage scale. Kruskal-Wallis and one-way ANOVA tests were used to compare the averages in three groups. Subsequently, to compare the two groups, the least significant difference (LSD) and Bonferroni post hoc tests were implemented. Accordingly, the Chi-square test was used to check the relationship between qualitative variables. In all analyses a p-value less than 0.05 was considered to be a significant difference. Pearson's correlation coefficient (r) and Spearman's rank correlation coefficient (ρ) were used to measuring the correlation between lipid profile, MMSE and CDR scores, $\Delta A\beta_{1-42}/A\beta_{1-40}$, and ΔsLRP1/sRAGE ratio. GraphPad Prism software (GraphPad Software; V9, San Diego, CA, USA) was used to draw the graphs and plots. In this study, the change in the $\Delta A\beta_{1-42}/A\beta_{1-40}$ level was considered as the primary outcome (Nakamura et al., 2018; Risacher et al., 2019; Turner et al., 2015) and MMSE and CDR scores as secondary outcomes (Turner et al., 2015). Also, adverse drug effects were recorded as another secondary outcome. The clinical effectiveness of the medication was determined to be 1.05 points of increase in the MMSE score between the groups, which is clinically significant (Birks and Harvey, 2018). CDR is reported in two forms, including the global score (G-CDR) (0–3 points) and the sum of boxes score (CDR-SB) (0–18 points), both of which represent the results of the six domains of the neurological clinical test (Andrews et al., 2019). A change of −1.63 point in the CDR-SB score, equivalent to −0.27 in the G-CDR score (Birks and Harvey, 2018; Andrews et al., 2019; Liu et al., 2021).

3. Results

The average age of the patients was 72.03±5.70. Twenty-five (69.4 %) women and eleven (30.6 %) men participated in this study. 66.7 % (n=24) lived with a partner and 33.3 % (n=12) lived alone. Most of the participants were housewives (41.66 %) or retired teachers (22.23 %). The duration of the disease was 3.03 ± 1.74 years and the average fasting blood sugar (FBS) of the patients was 97.9 ± 9.30 mg/dl. The average body mass index (BMI) of the patients was 26.64±2.73 and the systolic and diastolic blood pressures were 126.39±8.24 and 80.56 ±7.14 mm Hg, respectively.

3.1. Demographic characteristics in groups

At the baseline, no statistically significant difference was observed between the average age, clinical score of cognitive tests, and lipid profile level in the three groups. There was no statistically significant difference in the values of the variables at the baseline by the intervention groups. Likewise, the distribution of patients based on gender, a clinical score of cognitive tests, level of education, type of memory medication, or level of lipid profile did not have statistically significant changes (Table 1). There was no significant change between the groups in SBP, DBP, and FBS after the intervention (P>0.05). No significant changes were observed in BMI, ALT, and AST between study groups (P>0.05). The *posthoc* test showed a significant decrease in TC and LDL levels between the rosuvastatin group (-72.7±44.26 and −58.55±46, respectively) compared to the placebo (-10.9±39.02 and 0.57±44.54, respectively) (P=0.011 and P=0.026, respectively). Both the silymarin group (4.97±3.54) and the rosuvastatin group (4.80±0.12) showed a

Table 1
Comparison of baseline findings between patients after grouping.

Variables		Silymarin (n=12) Mean (SD)	Placebo (n=12) Mean (SD)	Rosuvastatin (n=12) Mean (SD)
age (years)		71 (6.46)	72.5 (6.02)	72.58 (4.85)
gender (percentage)	Female	66.7	66.7	75
	Male	33.3	33.3	25
Marital status (percentage)	with a partner	58.3	66.7	75
	alone	41.7	33.3	25
Duration of AD (years)		2.94 (2.48)	3.16 (1.17)	3 (1.45)
History of dyslipidemia (years)		5.34±0.72	4.94±1.38	5.72±1.09
Duration of medication (years)		1.29 (0.76)	1.3 (1.04)	1.68 (1.26)
MMSE score		20.5 (1.73)	20.67 (1.43)	21.17 (1.19)
ALT (U/L)		20.50 (8.33)	18.92 (6.99)	16.92 (7.20)
AST (U/L)		23.92 (6.05)	26.33 (9.57)	24.42 (5.23)
MDA (μM/L)		5.10 (1.16)	6.87 (3.41)	4.65 (1.83)
CAT (U/mL)		18.29 (3.33)	19.02 (5.85)	22.40 (7.24)
SOD (U/mL)		88.42 (10.68)	98.34 (13.38)	101.32 (17.80)

statistically significant increase in HDL when compared to the placebo group (-5.61±6.57) (P<0.000, P=0.044, respectively). The level of TG in the silymarin group (-71.45±24.59) has a significant decrease compared to both the placebo (-3.4±17.69) and the rosuvastatin group (-42.2±81.56) (P<0.000 and P =0.036) (Table 1 and Fig. 2).

3.2. Clinical outcomes and oxidative markers

Although there was no statistically significant difference between the groups in the MMSE score after the intervention (P>0.05), an increase of 1.73 points in the MMSE score of patients receiving silymarin compared to the placebo was seen, which is higher than the cut-off point and is considered clinically significant. Also, in the rosuvastatin group, the clinical score incremented by 1.68 points compared to the placebo. There is no statistically significant difference between the groups in the CDR score after the intervention (P>0.05). Also, a 0.2-point decrease in the CDR score of patients receiving silymarin compared to the placebo was seen, which was below the cut-off point defined for CDR and had no clinical significance. The level of MDA in the silymarin group (-2.11 ±0.93) compared to the rosuvastatin group (-0.47±1.26) has a significant decrease (P=0.005), but there is no statistically significant difference compared to the placebo (-4.11±0.82) (P= 0.171). Also, there is a significant increase in CAT level between the silymarin (9.94±6.68) and rosuvastatin (-4.13±7.53) group (P=0.0013). In addition, the SOD level in the silymarin group (41.98±13.96) compared to placebo (2.94 ±15.66) and rosuvastatin (12.38±15.54) had a significant increase (p<0.000 and p=0.008, respectively) (Fig. 3).

3.2.1. Amyloid peptides and its carrier

The ΔsLRP1/sRAGE ratio in the silymarin group (1.240±0.94) compared to the rosuvastatin (-0.348±0.584) group had a significant increase (p<0.000). There is a significant increase in the level of $\Delta A\beta_{1-42}/A\beta_{1-40}$ in the silymarin group (0.061±0.03) compared to the placebo (-0.005±0.49) and rosuvastatin (-0.028±0.15) (P=0.001 and P= 0.044, respectively) (Fig. 4). There is no significant relationship between the quadruple lipid profile (TC, TG, HDL, and LDL) with MMSE and CDR clinical tests (Table 2). Also, liver enzymes (ALT and AST) had no statistically significant relationship with the MMSE and CDR, MDA, and $\Delta A\beta_{1-42}/A\beta_{1-40}$. Furthermore, there was no statistically significant relationship between TC and LDL lipid factors with oxidative stress markers (MDA, CAT, and SOD), $\Delta A\beta_{1-42}/A\beta_{1-40}$, and ΔsLRP1/sRAGE. There is no significant relationship between liver enzymes and CAT, SOD, and ΔsLRP1/sRAGE (Table 2). Although there was a statistically inverse relationship between SOD level and ALT (r=-0.367 and P=0.042) (Fig. 5), there was no significant relationship with AST (r=0.009 and P=0.959). Although HDL was not correlated with MDA and CAT, it was statistically directly correlated with the SOD marker (r=0.412 and P=0.021) (Fig. 5). Although HDL was directly associated with the $\Delta A\beta_{1-42}/A\beta_{1-40}$ biomarker (ρ=0.379 and P=0.035), no association was observed between it and ΔsLRP1/sRAGE (Table 2 and Fig. 5). There is a direct and inverse relationship between TG and oxidative stress markers MDA and SOD, respectively (ρ=0.449 and P=0.011; ρ=-0.554 and P=0.001, respectively). In addition, there was an inverse relationship between TG and $\Delta A\beta_{1-42}/A\beta_{1-40}$ (ρ=-0.493 and P=0.004). Although the relationship between TG and ΔsLRP1/sRAGE was statistically significant (ρ=-0.392 and P=0.029) (Table 2 and Fig. 6). The relationship between multiple factors in the heat map is presented in Fig. 7. There is no significant relationship between oxidative stress markers (MDA, CAT, and SOD) and clinical tests (MMSE and CDR). On the other hand, $\Delta A\beta_{1-42}/A\beta_{1-40}$ has a direct statistical relationship with SOD marker (ρ=0.388 and p=0.031). Moreover, there was a direct correlation between the level of $\Delta A\beta_{1-42}/A\beta_{1-40}$ and ΔsLRP1/sRAGE (ρ=0.491 and P=0.005) (Fig. 7 and Table 3). Although there was no statistically significant relationship between CAT and $\Delta A\beta_{1-42}/A\beta_{1-40}$ (ρ=0.315 and P=0.083), ΔsLRP1/sRAGE had a direct relationship with this antioxidant (r=0.396 and P=0.027). There was a direct relationship between CAT and SOD (r=0.436 and P=0.014) but an inverse relationship between CAT and MDA (ρ=-0.409 and P=0.022) (Table 3).

3.3. Side effects reporting in groups

In the silymarin group, there were very few adverse events (AEs). A patient did report headache and dizziness, and another patient reported difficulty sleeping. Dizziness and appetite loss (30 %) was the most significant side effect in the rosuvastatin group. Then, among the other complaints made by patients in this group (20 %), were frequent urination, headaches, and irregular sleep patterns. In this group of patients, half experienced an appetite loss due to the placebo. Urinary disorders (40 %) characterized by burning sensation during frequent urination and diffuse headaches (30 %) were among the other complications associated with the placebo. No additional adverse effects, such as heart failure or eye disorders, occurred in any of the groups. During the study, one patient passed away after being admitted to the hospital with a femur fracture and COVID-19. During the intervention period, laboratory values of markers related to the blood, liver, and kidneys (e.g., RBC, WBC, Hgb, BUN, creatinine, and alkaline phosphatase) were recorded. No particular disorders were observed in any of these markers.

4. Discussion

The present study was conducted to investigate the effect of silymarin (compared to rosuvastatin) on blood markers and clinical status of mild Alzheimer's patients with secondary dyslipidemia. Our results revealed that there is a statistically significant decrease in serum levels of TC and LDL in the Rosuvastatin group compared to the placebo. The increase of LDL in the cell reduces the synthesis of HMG-CoA reductase, which decreases the intracellular synthesis of cholesterol. Because the interaction of amyloid and cholesterol is synergistic, the increase in Aβ leads to disruption in these regulatory mechanisms, and therefore, the increase in plasma cholesterol causes an increase in cellular cholesterol and amyloid deposition as well as disruption in the clearance of amyloid from the brain tissue. Since Aβ degradation and clearance are linked to cholesterol levels, these processes rely on lipid rafts rich in cholesterol and proteins like ApoE and ABC pumps that transport cholesterol. So it seems cholesterol has a dual impact on Aβ toxicity or clearance, as it can both encourage and impede Aβ aggregation at the membrane. This, in turn, affects the secondary structure of amyloid and its capacity to

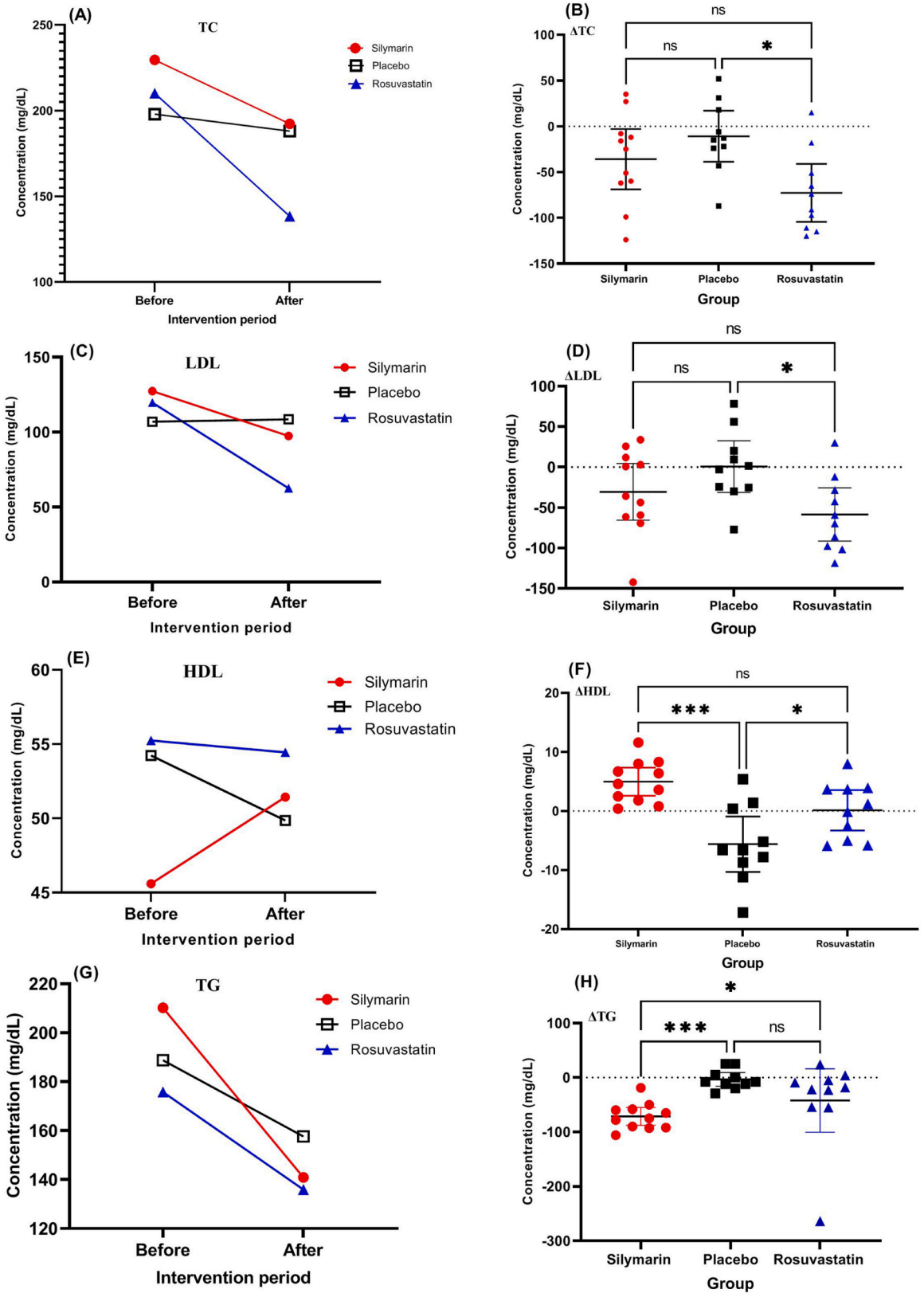

Fig. 2. A, C, E, and G show mean±SD of TC, LDL, HDL, and TG levels at the beginning and end of the intervention within the groups, respectively. B, D, F, and H show the delta (difference) after 6 months of intervention, between the groups, based on the mean with a 95 % confidence coefficient (CI 95 %). * $P<0.01$ and *** $P<0.0001$.

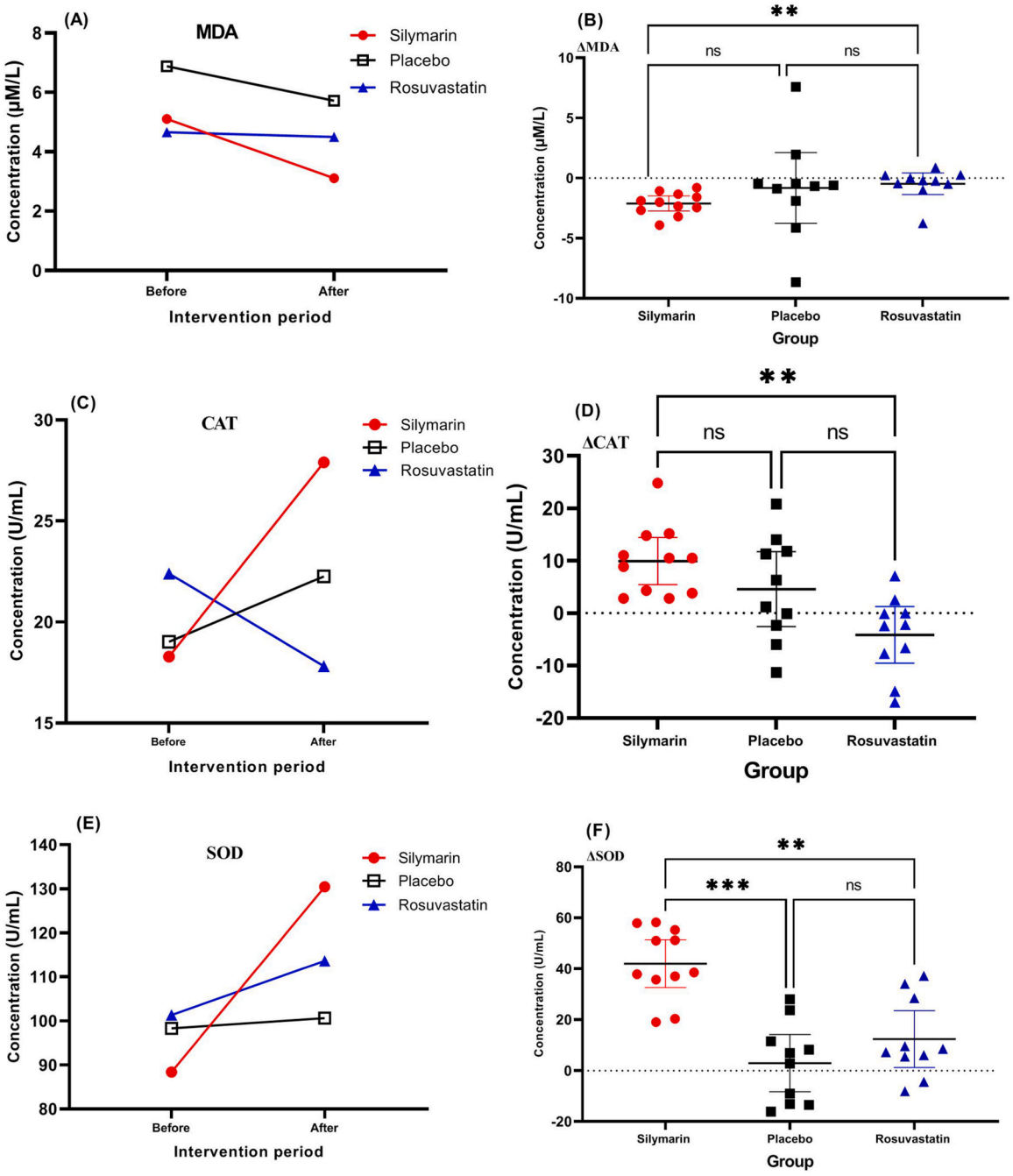

Fig. 3. A, C, and E, show mean±SD of MDA, CAT, and SOD levels at the beginning and end of the intervention within the groups, respectively. B, D, and F show the delta (difference) after 6 months of intervention, between the groups, based on the mean with a 95 % confidence coefficient (CI 95 %). ** P<0.001 and *** P<0.0001. Although the level of MDA and CAT in the silymarin group compared to only the rosuvastatin group has a significant decrease and increase, the SOD level of the silymarin group has a significant increase compared to both the placebo and rosuvastatin groups.

interact with the cell membrane (Rudajev and Novotny, 2022) which our findings support this issue. Excess cholesterol is removed by HDL, which is considered a cholesterol regulator (Feingold, 2022). Although a robust association between hypercholesterolemia and AD has been shown, cholesterol itself is unable to cross the BBB into the brain. Cholesterol-lowering therapies have shown paradoxical results on cognitive function in AD patients, raising the question of whether cholesterol metabolism in the brain should be separate from peripheral cholesterol metabolism. Hypercholesterolemia leads to increased levels of 27-Hydroxycholesterol (27-HC) in the brain and circulation and affects the metabolism and regulation of cholesterol, which is considered one of the reasons for the rapid progression of AD. Because the plasma levels of the main circulating oxysterols (24-HC and 27-HC) were significantly higher in AD patients compared to healthy subjects (Choroszyński et al., 2022). Studies show that oxidative stress, inflammation, and altered cholesterol metabolism contribute to the development of AD (Gamba et al., 2015). 27-HC is significantly absorbed from the circulation into the brain (Gamba et al., 2012), which may be the missing link between cholesterol disorders and AD (Merino-Serrais et al., 2019). It has also been suggested that 27-HC is the main cause of memory impairment caused by blood and dietary cholesterol (Jahn et al., 2021). Moreover, 27-HC leads to increased production and accumulation of Aβ and hp-tau and AD progression, which can be modulated via the pleiotropic effects of lipid-lowering drugs

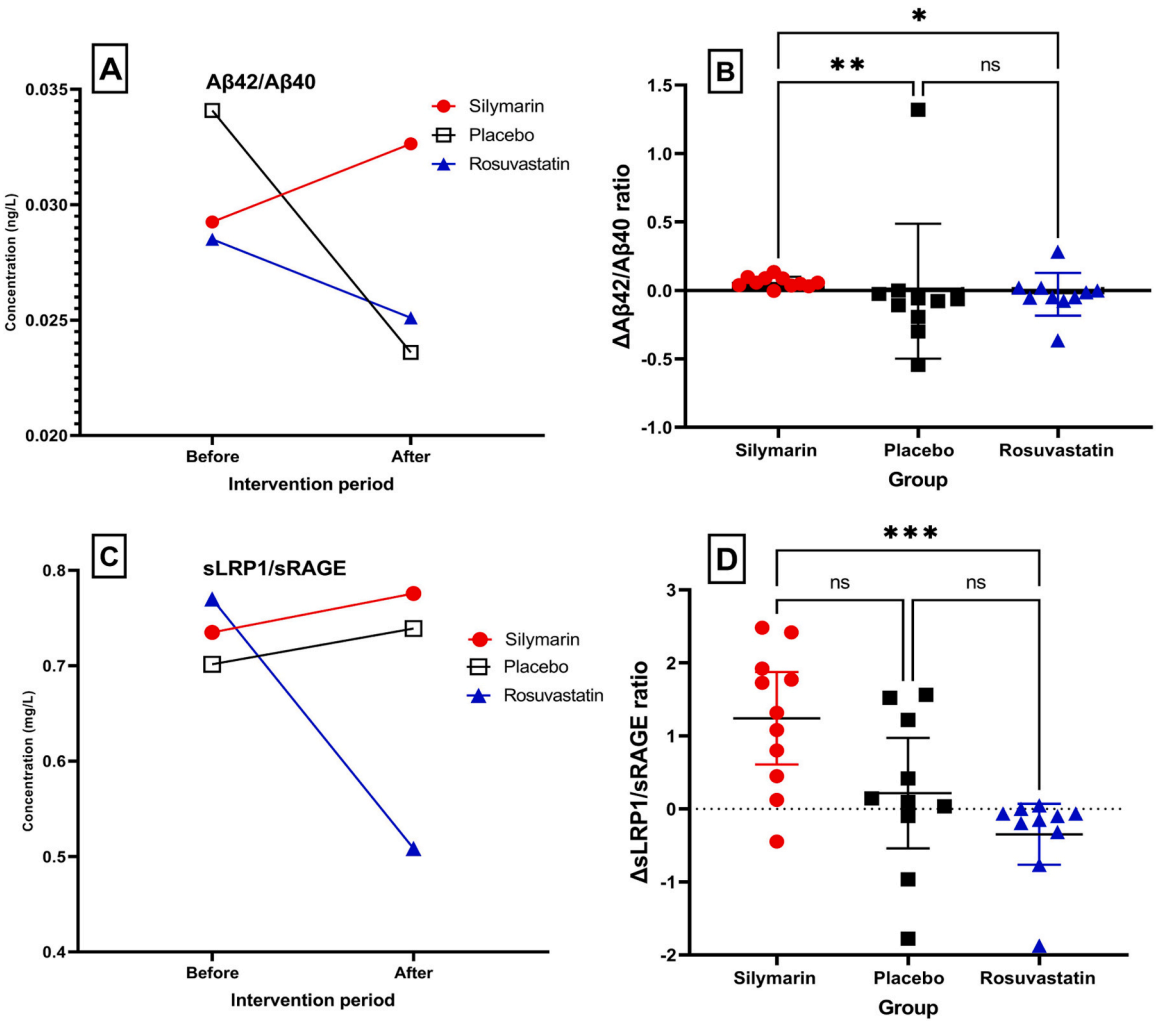

Fig. 4. A and C show mean $A\beta_{42}/A\beta_{40}$ and sLRP1/sRAGE levels at the beginning and end of the intervention within the groups, respectively. B and D show the delta (difference) after 6 months of intervention, between the groups, based on the mean with a 95 % confidence coefficient (CI 95 %). * P<0.01, ** P<0.001 and *** P<0.0001.

Table 2
Correlation results of the difference of lipid profile and liver enzymes with markers of oxidative stress, amyloid-beta, and its carrier with clinical results.

Variables	ΔLDL		ΔHDL		ΔTG		ΔTC	
	correlation strength (r)	significance (p)	correlation strength (r)	significance (p)	correlation strength (r)	significance (p)	correlation strength (r)	significance (p)
ΔMDA	−0.182	0.325	−0.255	0.165	0.449	0.011	−0.157	0.396
ΔCAT	−0.013	0.940	0.298	0.102	−0.314	0.084	0.030	0.871
ΔSOD	−0.089	0.633	0.412	0.021	−0.554	0.001	−0.080	0.667
$\Delta A\beta_{42}/\Delta A\beta_{40}$	−0.136	0.462	0.379	0.035	−0.493	0.004	−0.106	0.569
ΔsLRP1/ sRAGE	−0.320	0.079	0.156	0.400	−0.392	0.029	0.306	0.093

(Loera-Valencia et al., 2019).

The significant increase in HDL in the silymarin and rosuvastatin group compared to placebo in our study was consistent with the results of the study by Sobolova *et al* (Sobolová et al., 2006).. HDL prevents LDL oxidation, regulates cholesterol circulation, alleviates atherosclerotic plaques, and decreases the risk of AD (Liu et al., 2020). Nunes *et al.* (2014) showed that plasma 27-HC was increased in low HDL than in high HDL subjects (Nunes et al., 2014). Furthermore, cell cholesterol efflux rate was reduced in subjects with low HDL compared to the group with high HDL, which may be demonstrating an alternative pathway (particularly involving the amyloid deposition complex (Michikawa et al., 2001) to maintain appropriate cellular cholesterol levels (Nunes et al., 2014). Our study showed that TG in the silymarin group was significantly attenuated compared to the placebo and rosuvastatin groups. High cholesterol levels directly affect the beta/gamma-secretase cleavage, and lead to its accumulation and aggregation in the extracellular space (Wingo et al., 2019). The study of Bowman et al. showed that dyslipidemia leads to brain inflammation and changes in the integrity of the BBB, and reducing TG and raising HDL can play an important role in modulating the pathological changes of AD patients from mild to moderate stage (Bowman et al., 2012). Therefore, it can be concluded that dyslipidemia, especially the abnormal changes of HDL and TG, is an important factor in the progression of AD and lack of response to treatment in these patients, so we call it the peripheral lipidopathy

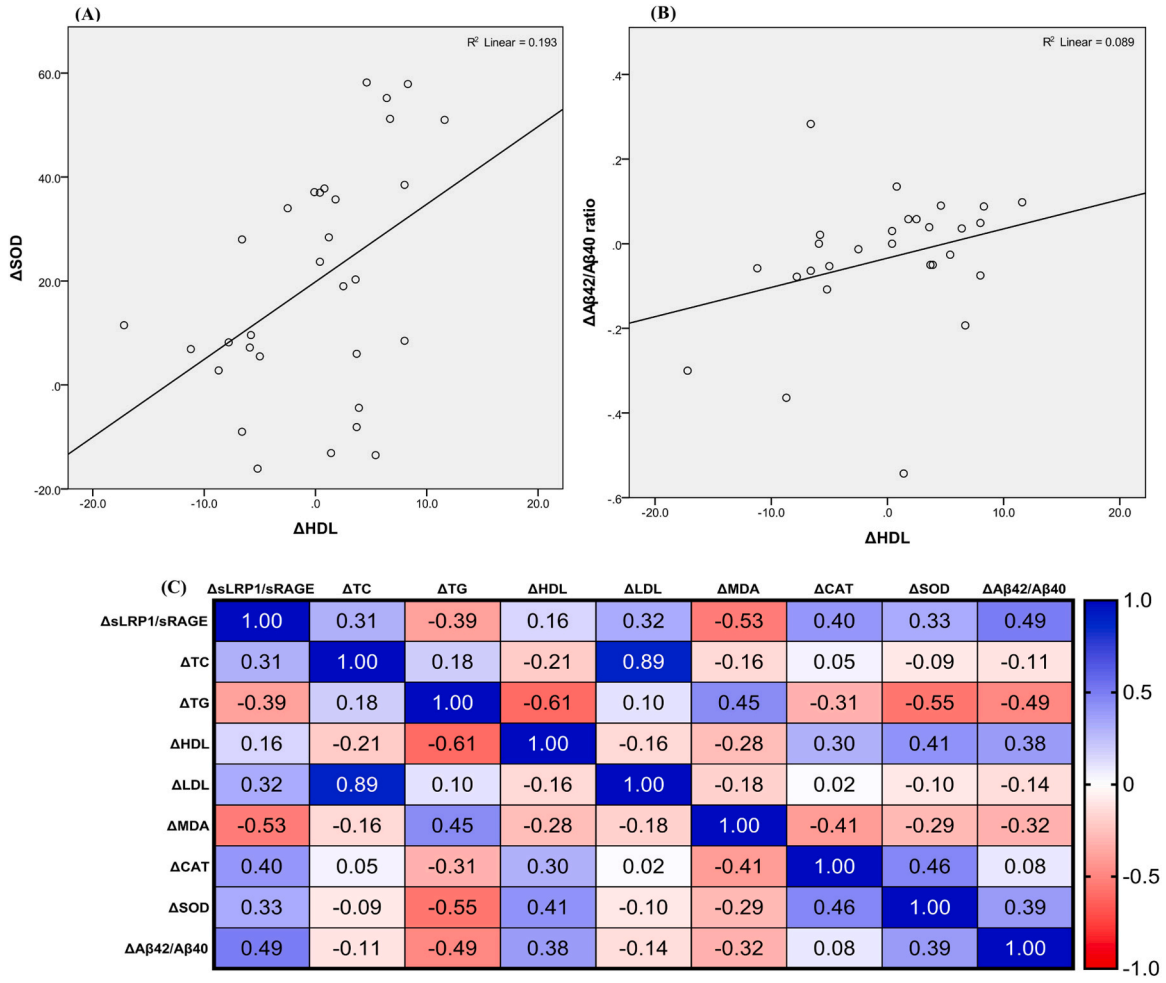

Fig. 5. ΔHDL factor has a direct relationship with ΔSOD (A) and $\Delta A\beta_{1-42}/A\beta_{1-40}$ (B) markers. Heat map showing the relationship between multiple serum factors with HDL and with each other. The red color indicates an inverse relationship and the blue color indicates a direct relationship (C).

hypothesis.

Moreover, several clinical studies show that donepezil, although it reduces the activity of AChE, decreases the Aβ accumulation, and improves the cognitive status and memory (Kume et al., 2005), it abnormally alter lipid profile in AD patients (Cacabelos, 2007). It seems that some fluctuations in serum/plasma biomolecule levels in abnormal conditions can cause significant changes in brain structural and functional plasticity, which may be caused by the activation of compensatory mechanisms that have been reported in many brain disorders (Heidari et al., 2023, 2020, 2017a, 2017b). In our study, patients receiving silymarin had lower TG levels and higher HDL levels, and significant reductions in TC and LDL were observed in the rosuvastatin group, demonstrating the importance of combination therapy in AD patients. This approach not only preserves donepezil's beneficial properties (anti-Aβ and AChE inhibitory effects), but also prevents its disruptive effects on lipid metabolism. Pattanashetti *et al.* reported that combination therapy with quercetin and donepezil has a synergistic effect and improves cognitive memory by reducing the AchE and $A\beta_{1-42}$ and increasing the activity of the antioxidant system in the rat brain (Pattanashetti et al., 2017). Our findings showed that silymarin enhanced the cognitive effect of donepezil by modulating the lipid profile, increasing $\Delta A\beta_{1-42}/A\beta_{1-40}$ and antioxidant activities, which is consistent with the study of Pattanashetti et al. (2017).

In the present study, MDA and CAT levels in the silymarin group decreased and increased significantly compared to the rosuvastatin group, respectively, but the SOD level in the silymarin group had a statistically significant increase compared to both placebo and rosuvastatin groups. SOD and CAT are enzymatic antioxidants that act as free radical scavengers, thereby acting as the first line of defense against ROS-induced damage (Varesi et al., 2023). Lipid peroxidation is an early key event in AD, preceding amyloid deposition and NFT formation.

Exposure of lipids to free radicals increases ROS levels, therefore, leading to enhancing gamma-secretase activity (Pratico et al., 2001; Gwon et al., 2012). In this regard, studies have revealed that Aβ accumulation occurs more easily in membranes composed of oxidized lipids and unsaturated lipids are more vulnerable to oxidative stress (Koppaka and Axelsen, 2000). Increased expression of SOD leads to a reduction of oxidative activity and amyloid plaque in the hippocampus and an improvement of memory deficit in the Alzheimer's model (Massaad et al., 2009). Flavonoids activate the PI3K/Akt pathway, which results in the reduction of amyloid plaque and lipid peroxidation, an increase of Ach level, inhibition of hp-tau, enhancement of SOD, and CAT activity (Varesi et al., 2023; Arslan et al., 2020). Silymarin has been shown to have a modulating effect on this pathway, inhibiting AChE and reducing the accumulation of Aβ peptide (Duan et al., 2015; Haddadi et al., 2020). In addition, El-Marasy *et al.* (2018) demonstrated that in a rat model of dementia, the administration of 400 mg/kg silymarin along with 10 mg/kg donepezil for 14 days diminished memory decline, as well as activity of AChE and MDA, while increasing levels of Glutathione (GSH) in the hippocampus. They reported that pretreatment with silymarin restores GABA, acetylcholine, and dopamine contents similar to donepezil. This shows that the antioxidant effect of silymarin preserves neurotransmitters and improves memory function in rats (El-Marasy et al., 2018). Our findings are consistent with those of some of these

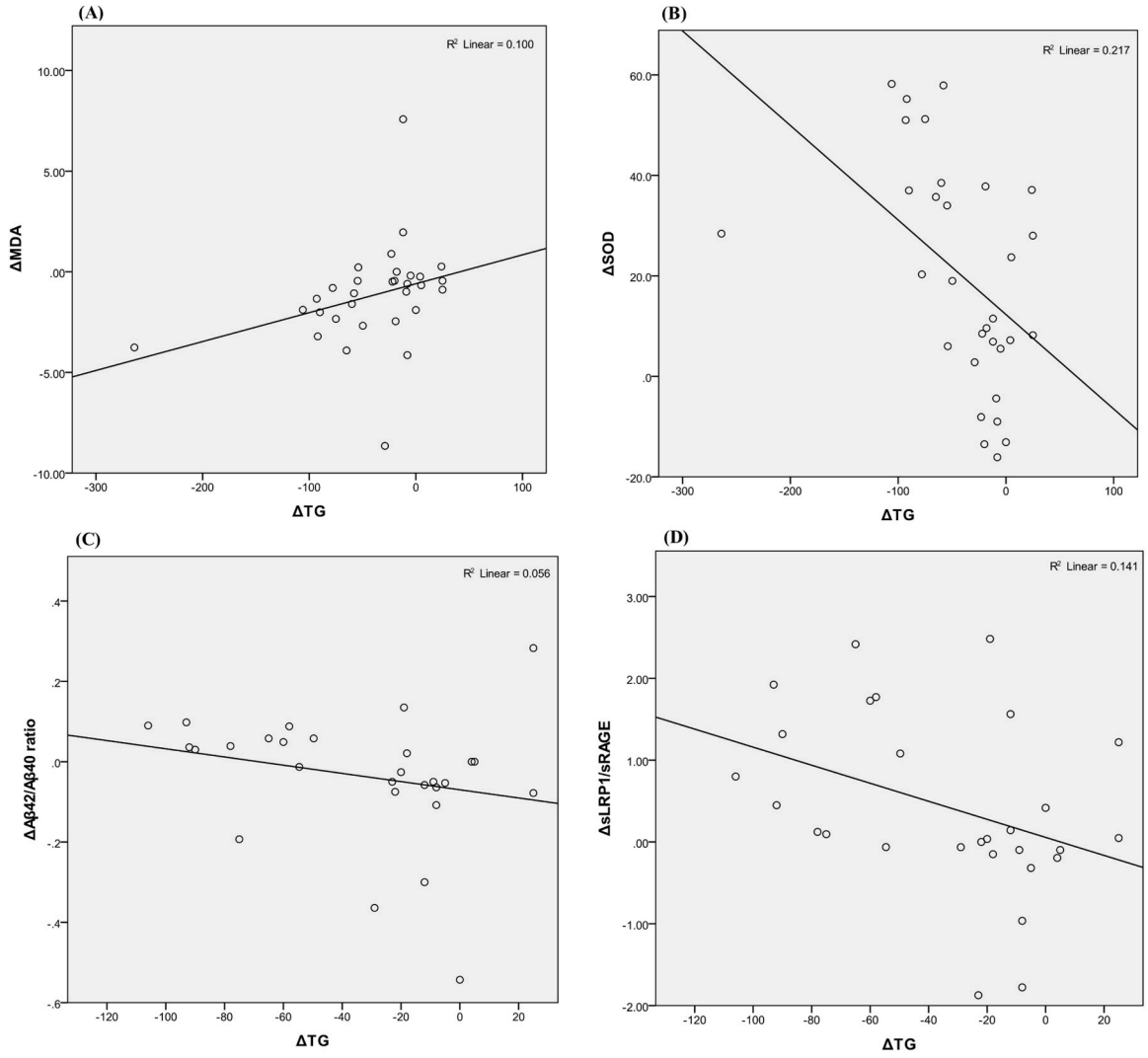

Fig. 6. The direct and inverse correlation between ΔTG and the ΔMDA and ΔSOD markers (A and B), respectively. There was also an inverse correlation between ΔTG and $\Delta A\beta_{1-42}/A\beta_{1-40}$ and ΔsLRP1/sRAGE (C and D).

studies such as modulating oxidative stress markers and increased peripheral clearance of Aβ. The non-significance of the effect of silymarin on MMSE and CDR scores can probably be attributed to the small sample size and the short duration of patient follow-up.

LRP1 has been shown to remove Aβ from the interstitial fluid (ISF) by endocytosis or lysosomal degradation in or across BBB cells (Li et al., 2001). Dysfunction of capillary endothelial cells leads to incomplete clearance of Aβ at the BBB and results in Aβ deposition in the brain, which is the basis of neurodegeneration and cognitive impairment (Zhang et al., 2022). A study by Wang *et al.* showed that silybin, a polyphenolic compound of silymarin, has a protective effect on endothelial cells (Wang et al., 2005). Palomino *et al.* reported that 100 μg/mL silymarin protects endothelial cells from oxidative damage by modulating antioxidant enzyme activity (Palomino et al., 2017). In our study, the increase in the level of ΔsLRP1/sRAGE ratio is probably due to the effects of silymarin on the expression of LRP1 in endothelial cells, but more gene expression and immunohistochemical studies are needed.

Dyslipidemia is associated with BBB disruption in AD patients, and plasma TG and HDL cholesterol are the most important factors involved in maintaining BBB integrity in these patients (Bowman et al., 2012; Acharya et al., 2013). The study of Bowman *et al.* revealed that dyslipidemic AD patients have significantly high plasma levels of TG and low HDL, and BBB disruption is common in them (Bowman et al., 2012). Dyslipidemia impairs Aβ clearance by reducing LRP1 expression in cerebrovascular endothelial cells, which results in decreased peripheral clearance of amyloid and leads to Aβ accumulation in the form of senile plaques (Prasanthi et al., 2008). In addition to causing neurodegeneration and reduced cognitive capacity, dyslipidemia also has detrimental effects on the cardiovascular system, which could be an overlapping mechanism in the amyloid hypothesis (Stakos et al., 2020; Waigi et al., 2023). It has been reported that the reduction of LRP1 in vascular smooth muscle cells leads to brain hypoperfusion and hypoxia, which in feedback leads to further reduction of LRP1 serum level (Nelson et al., 2016). Therefore, the increase in the serum level of ΔsLRP1/sRAGE ratio in the silymarin group in our study shows that this peptide probably mediates the uptake and degradation rate of Aβ in vascular smooth muscle cells and plays an essential role in Aβ clearance and improves the cognitive status of patients (Van Gool et al., 2019). The low concentration of $A\beta_{1-42}$ in blood circulation indicates the increase of Aβ in the brain, the formation of amyloid plaques, and cognitive deficits in AD patients (Risacher et al., 2019; Pérez-Grijalba et al., 2019; Cianflone et al., 2021). A prospective study by Lambert *et al.* (2009) in France showed that individuals with high plasma $A\beta_{1-42}/A\beta_{1-40}$ levels had a lower risk of developing dementia and concluded that plasma Aβ concentration may be a valuable marker for screening and diagnosis of these patients (Lambert et al., 2009). Nakamura *et al.* showed that Aβ biomarker levels in three sites including circulation, CSF, and PET imaging of the cortex were highly correlated with each other, clearly

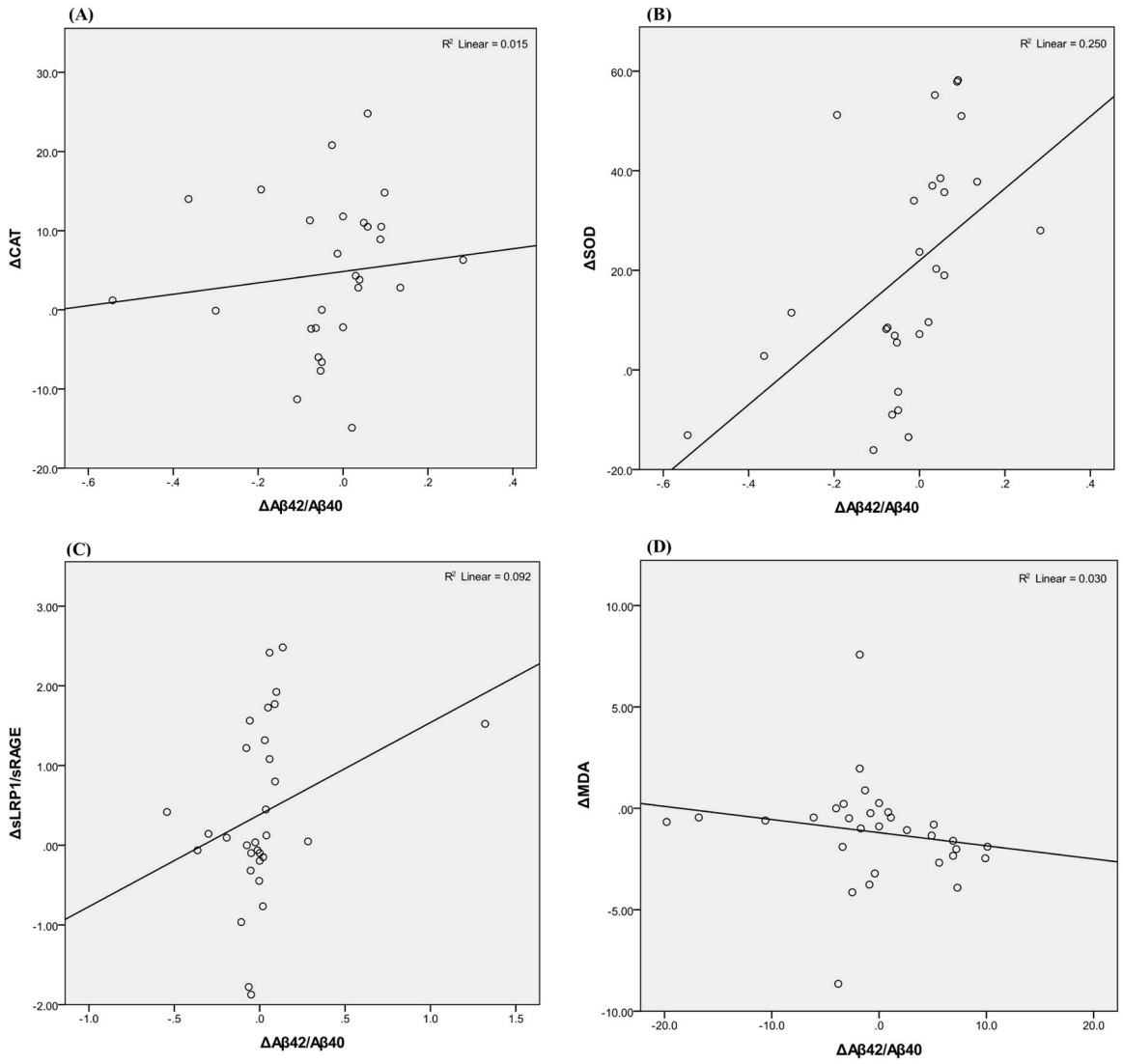

Fig. 7. The $\Delta A\beta_{1-42}/A\beta_{1-40}$ marker has no statistically significant relationship with CAT marker (A). There is a statistical inverse, a direct and direct correlation between $\Delta A\beta_{1-42}/A\beta_{1-40}$ and MDA, SOD, and ΔsLRP1/sRAGE markers, respectively (B, C, and D).

Table 3
Correlation results of differences of oxidative stress markers, amyloid-beta and its carrier with clinical results.

Variables	ΔsLRP1/sRAGE		$\Delta A\beta_{42}/\Delta A\beta_{40}$		ΔSOD		ΔCAT		ΔMDA	
	correlation strength (r)	significance (p)	correlation strength (r)	significance (p)	correlation strength (r)	significance (p)	correlation strength (r)	significance (p)	correlation strength (r)	significance (p)
ΔMDA	−0.345	0.056	−0.369	0.041	−0.290	0.113	−0.409	0.022	1	-
ΔCAT	0.441	0.012	0.315	0.083	0.436	0.014	1	-	−0.409	0.022
ΔSOD	0.427	0.016	0.722	<0.000	1	-	0.436	0.014	−0.290	0.113
$\Delta A\beta_{42}/\Delta A\beta_{40}$	0.491	0.005	1	-	0.388	0.031	0.084	0.650	−0.320	0.079
ΔsLRP1/sRAGE	1	-	0.491	0.005	0.328	0.071	0.396	0.027	−0.533	0.002

indicating that plasma Aβ level is strongly related to Aβ status in the CNS. Since the plasma $A\beta_{1-42}$ assay has an accuracy of 80.4 % in the diagnosis and monitoring of AD patients, it can reduce the cost of unnecessary PET-Aβ scans and be significantly useful in clinical program decision-making (Nakamura et al., 2018). Andersson *et al.* revealed that the Aβ42/Aβ40 ratio in CSF and serum significantly decreased with age in an Alzheimer's mouse model, which was associated with increased Aβ plaque burden in the brain (Andersson et al., 2023).

Risacher *et al.* suggested that plasma Aβ measurement can be a useful biomarker for predicting brain Aβ burden in AD patients, as lower plasma Aβ42/40 levels are associated with high cortical Aβ deposits, thus differentiating between healthy and MCI subjects (Risacher et al., 2019). In our study, a significant increase in serum levels of $A\beta_{1-42}$ was observed in the silymarin group compared to the placebo and rosuvastatin groups. Wei *et al.* revealed that 40 mg/day of simvastatin for 12 weeks could significantly increase the plasma level of $A\beta_{1-42}$ in hyperlipidemic patients compared to placebo. They reported that the increase in plasma $A\beta_{1-42}$ was directly related to the decrease in TG levels and

RAGE (a peptide that acts opposite to LRP1 and promotes amyloid transport from the circulation to the brain), which indicates that statins play a role in the transfer of Aβ to plasma and its peripheral clearance (Wei et al., 2022). The results of our study demonstrated that silymarin, as a lipid-lowering compound, increases the serum level of ΔsLRP1/sRAGE ratio and $\Delta A\beta_{1-42}/A\beta_{1-40}$, as well as decreases the level of TG, which is in line with the study of Wei *et al.*. Although there was no statistically significant difference in MMSE scores between groups, in terms of clinical efficacy, a 1.73-point increase in MMSE score was observed in patients receiving silymarin compared to placebo. Also, in the rosuvastatin group, the MMSE score improved by 1.68 points compared to the placebo.

Clinical trials have shown that AChE inhibitors, particularly donepezil, for 6 months in Alzheimer's disease increases the MMSE score by 1.73 points (Perera et al., 2014) and it has been proven that silymarin is an effective AChE inhibitor (Duan et al., 2015). A Cochrane study by Birks showed that the once-daily administration of 10 mg/kg donepezil over 26 weeks resulted in a 1.1-point change in the MMSE score of mild AD patients compared to placebo, which is clinically considered to improve cognitive function (Birks and Harvey, 2018). In our study, although there was no statistical correlation between TC and LDL lipid factors with MMSE and CDR tests, oxidative stress markers (MDA, CAT, and SOD), $\Delta A\beta_{1-42}/A\beta_{1-40}$, and ΔsLRP1/sRAGE ratio, the clinical efficacy of silymarin and rosuvastatin compared with placebo, it may have the neurological outcome in the clinic and will be better understood in the future by designing long-term studies with a larger sample size. Studies show that although treatment may have no obvious clinical benefit (because symptoms only stabilize), the patient's condition worsens if treatment is stopped (Knowles, 2006). Miyakawa *et al.* reported that AD patients experienced an average yearly decline of 2.43 points in the MMSE score (Miyakawa-Liu et al., 2022). To conclude with 90 % confidence that an Alzheimer's patient has experienced a "true" clinical change, one should expect changes of 2–4 points in the MMSE score to occur after 1.5 years because smaller changes are interpreted with uncertainty. Sometimes patients may show a true change, but the patient's symptoms are interpreted with insufficient certainty (less than 90 %) (Hensel et al., 2007). Because the MMSE score at baseline is a suitable predictor for assessing the response to AChE inhibitors (Lopez et al., 2010), so considered a practical tool for assessing cognitive improvement and evaluating the progress and severity of dementia (Arevalo-Rodriguez et al., 2015) and has acceptable validity and reliability in measuring the outcome (Knowles, 2006). Studies show that the CDR-SB score (18 points) in mild AD patients who do not use disease-specific drugs (donepezil, etc.) increases annually between 1.43 and 2.4 points (equivalent to 0.23–0.4 points in the G-CDR score) (Samtani et al., 2014; Williams et al., 2013; Berg et al., 1992). In our study, there was no statistically significant decrease in the CDR score. In terms of minimal clinically important difference (MCID), the reduction of CDR in the silymarin and rosuvastatin groups compared to the placebo was lower than the clinical efficacy endpoint, which was probably due to the short duration of the study.

5. Conclusion

This study showed the relationship between plasma lipids, especially TG and HDL, with amyloid clearance in dyslipidemic AD patients with mild dementia and proved that the modulation of these lipid factors can be used as easily accessible biomarkers to monitor the response to treatments. Donepezil combined with silymarin was effective in improving the serum characteristics of mild dyslipidemic AD patients, especially improving the lipid profile, enhancing amyloid clearance, ΔsLRP1/sRAGE ratio, and improving antioxidant enzymes after 6 months. Further studies are needed to find out the exact signaling pathways. HDL, TG, and sLRP1 may influence AD pathological processes through multiple mechanisms such as Aβ processing, synaptic function, hp-tau, and peripheral clearance dependent on transport across the BBB. The physical interaction between sLRP1 and Aβ, TG and Aβ or HDL and Aβ in the presence of silymarin and rosuvastatin may be a key project to investigate the mechanisms of AD pathogenesis, better to use well-controlled genetic animal models to get a clearer picture of what occurs in the brain and blood circulation. In the end, considering the complexity of AD and multiple factors in its occurrence and progression, clinical trial studies in this field should be based on accessible and sensitive diagnostic methods, such as the measurement of serum $A\beta_{1-42}$ consensus biomarkers, MRI morphometric imaging, and neuropsychological tests so that their results can be used in the design of future studies.

6. Limitations of the study

Among the problems, the COVID-19 pandemic and the imposed restrictions were one of the obstacles to the progress of the project, and their coordination for screening was very time-consuming. Due to the high cost of PET-Aβ, the presence of 5 PET machines in Iran, and long admission times due to the coronavirus, we could not investigate the effects of environmental Aβ clearance on brain Aβ accumulation. One of the limitations of our study was the lack of applying multiplicity adjustment to the multiple secondary outcome analyses because the error variance of the dependent variable according to Levene's test was not equal across groups (homogeneity of variance). Therefore, we could not perform ANCOVA statistical analysis.

Ethics approval and consent to participate

This study was conducted based on Good Clinical Practice (GCP) guidelines by considering Helsinki Declaration. This study was conducted under the supervision of the Iran University of Medical Sciences (IUMS) Ethics Committee IR.IUMS.FMD.REC.1400.409 and Iran Clinical Trial Registration Center (approved IRCT code: IRCT20210901052360N1). In this study, the confidential and personal information of the patient, as well as, the psychological safety of the patient were preserved privately. The written consent obtained from the participants or their legal representatives was approved by the ethics committee of the Iran University of Medical Sciences. The written consent obtained from the participants or their legal representatives was approved by the ethics committee of the Iran University of Medical Sciences.

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CRediT authorship contribution statement

Zahra Ghobadi: Data curation, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Fatemeh Moradi:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Auob Rustamzadeh:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Software, Writing – original draft, Writing – review & editing. **Fatemeh Khamseh:** Data curation, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nafiseh Mohebi:** Data curation, Investigation, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nader Sadigh:** Conceptualization, Data curation, Formal analysis, Supervision, Validation, Writing – original draft, Writing – review & editing. **Zahra Vahabi:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The Authors declare no competing financial or non-financial interests directly or indirectly related to the work submitted for publication.

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Consent for publication

All authors gave consent for publication.

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