

Original Article

Atractylenolide I mitigates Alzheimer's disease pathology in ApoE^{-/-} mice via ARG1/nNOS axis and lipid homeostasis regulation

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Abstract

Apolipoprotein E (ApoE) serves as a critical molecular nexus between Alzheimer's disease (AD) and atherosclerosis, two age-associated inflammatory disorders that share vascular pathology, amyloid-beta (Aβ) deposition, and lipid dysregulation. Atractylenolide I (AI), a promising therapeutic candidate derived from *Atractylodes macrocephala* Koidz., exhibits multimodal bioactivities with demonstrated anti-inflammatory and neuroprotective properties. To explore its therapeutic potential against AD pathology, we use high-fat diet (HFD)-fed ApoE knockout (ApoE^{-/-}) mice treated with or without AI for 12 weeks. Integrated bioinformatics analyses and experimental validation reveal that AI treatment markedly attenuates systemic lipid dyshomeostasis, particularly cerebral lipid deposition, suppresses neuroinflammation via downregulation of M1 macrophage polarization markers, and restores cognitive function through neuronal preservation in hippocampal regions. Mechanistically, AI orchestrates cholesterol efflux by up-regulating ATP-binding cassette transporter A1 (ABCA1) and liver X receptor (LXR) expression, while concurrently modulating the abundance of arginine biosynthesis metabolites (urea, malic acid, and creatinine) to rebalance neurovascular homeostasis. Notably, western blot and RT-qPCR analyses reveal that AI differentially regulates key enzymes including arginase 1 (ARG1) and simultaneously upregulates the expression of neuronal nitric oxide synthase (nNOS). Further molecular docking and surface plasmon resonance (SPR) analyses confirm the direct binding of AI to ARG1, indicating a novel neuroprotective mechanism involving the modulation of arginine metabolism. These findings delineate the pleiotropic effects of AI against AD pathology and establish a preclinical foundation for the development of AI-based therapeutics targeting neurodegenerative-cardiovascular comorbidities.

Key words Alzheimer's disease, atractylenolide I, lipid homeostasis, arginine biosynthesis, ApoE^{-/-} mice

Introduction

Alzheimer's disease (AD), the most prevalent neurodegenerative disorder, is characterized by the progressive accumulation of amyloid-β (Aβ) plaques and intraneuronal neurofibrillary tangles (NFTs), both of which are pathological hallmarks associated with chronic neuroinflammation and sustained neuronal degeneration [1]. In turn, atherosclerosis, a systemic inflammatory vasculopathy,

shares overlapping pathophysiological mechanisms with AD, particularly their mutual association with Aβ metabolism and apolipoprotein E (ApoE) polymorphism [2,3]. The polymorphic ApoE protein, which exists as three major isoforms (ε2, ε3, and ε4), plays critical roles in cholesterol homeostasis, blood-brain barrier (BBB) integrity maintenance, and Aβ clearance, thereby implicating it in both AD pathogenesis and atherosclerotic progression [4–7].

Notably, transgenic models expressing human amyloid precursor protein (APP) variants exhibit accelerated atherogenesis, revealing mechanistic intersections between cerebral amyloidosis and vascular inflammation [8]. Genome-wide association studies further substantiate molecular crosstalk between these conditions through shared susceptibility loci [9]. Emerging evidence implicates A β -mediated macrophage activation as a critical amplifier of neurovascular inflammation. Specifically, cytotoxic A β species activate cerebral macrophages, triggering self-perpetuating neuroinflammation via cytokine overproduction and neuronal apoptosis [10].

Macrophage polarization dynamics underpin this pathological crosstalk [11,12]. Proinflammatory M1 polarization, characterized by increased tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and IL-1 β secretion, as well as inducible nitric oxide synthase (iNOS) overexpression [13,14], exacerbates both neurotoxicity and vascular inflammation. Liver X receptors (LXRs) orchestrate inflammatory resolution through the dual regulation of cholesterol efflux (via ABCA1 induction) and the suppression of proinflammatory cytokines [15,16], emerging as key modulators of macrophage immune-metabolism [17]. Arginase 1 (ARG1), a cytoplasmic enzyme, acts as a pivotal regulator of macrophage functionality by regulating L-arginine metabolism, nitric oxide (NO) generation, inflammation, and oxidative stress. Neuronal nitric oxide synthase (nNOS), the primary NO-producing enzyme in the brain, mediates various functions, including learning, memory, synaptic plasticity, and neuronal development. It is also implicated in AD, psychiatric illnesses, and cognitive deficits [18]. The ARG1/NOS axis critically balances inflammatory responses through competitive substrate utilization, while ARG1 catalyzes the conversion of L-arginine to ornithine, and nNOS governs nitric oxide production that is essential for neurovascular homeostasis [19]. Dysregulation of this metabolic axis correlates with accelerated atherosclerosis and AD progression, manifesting as reduced nNOS expression in atherosclerotic plaques [20] and cognitive impairment [21,22].

Atractylenolide I (AI), a bioactive sesquiterpenoid isolated from *Atractylodes macrocephala* Koidz., demonstrates multitarget anti-inflammatory effects [23,24] against atherosclerosis [25]. Despite its known suppression of M1 markers (TNF- α /iNOS) in lipopolysaccharide-stimulated macrophages [26], the mechanisms through which AI alleviates AD and atherosclerosis remain unexplored. In the present study, we investigated the dual neurovascular protective effects of AI in an ApoE^{-/-} mouse model and demonstrated its ability to ameliorate lipid homeostasis and mitigate inflammatory cascades. Through integrative omics analyses and molecular docking validation, we identified ARG1 as a pivotal molecular target of AI, establishing a novel regulatory mechanism through which AI modulates arginine biosynthesis to combat neurodegenerative pathology.

Materials and Methods

Chemicals and reagents

Atractylenolide I (A0372; Munster, Chengdu, China) and a 60 kcal% fat diet (D12492; Research Diets, New Brunswick, USA) were used in the experiments. The following assay kits were also used: TNF- α kit (JL10484; J&L Biological, Shanghai, China), IL-6 kit (JL20313; J&L Biological), nNOS kit (BY-EM224003; BYabscience, Nanjing, China), total cholesterol (T-CHO) assay kit (A111-1-1; Nanjing Jiancheng, Nanjing, China), triglyceride (TG) assay kit (A110-1-1;

Nanjing Jiancheng), low-density lipoprotein cholesterol (LDL-C) kit (A113-1-1; Nanjing Jiancheng), and high-density lipoprotein cholesterol (HDL-C) kit (A112-1-1; Nanjing Jiancheng). The antibodies used included rabbit anti-ZO-1 (13663S; CST, Boston, USA), rabbit anti-OCN (91131S; CST), rabbit anti-Bax (5023S; CST), mouse anti-B-cell lymphoma-2 (Bcl-2; 15071S; CST), rabbit anti-Caspase-3 (14220S; CST), mouse anti-Caspase-9 (9508S; CST), rabbit anti-iNOS (BA0362; Boster, Wuhan, China), rabbit anti-IL-1 β (31202S; CST), rabbit anti-LXR (ab21669; Abcam, Cambridge, UK), rabbit anti-ABCA1 (BA1541-2; Boster), rabbit anti-ARG1 (93668S; CST), rabbit anti-nNOS (4231S; CST), rabbit anti- β -Actin (4970; CST), and rabbit anti- α -Tubulin (2125S; CST).

Ethics statement

Animal experiments were carried out according to regulations outlined in the Administration of Experimental Animals issued by the Ministry of Science and Technology of the People's Republic of China. This study was approved by the Animal Experimental Center of Guangzhou University of Traditional Chinese Medicine, and stringent adherence to guidelines for ethical treatment was maintained (animal experiment ethical number: 20230719001; date: July 19, 2023).

Animals

Male C57BL/6J mice (8 weeks) and ApoE knockout C57BL/6J mice (ApoE^{-/-} mice) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All animals were housed in the Animal Laboratory Center of Guangzhou University of Traditional Chinese Medicine under a 12-h light/dark cycle in a specific pathogen-free (SPF) environment with controlled temperature (22–24°C) and humidity (50%–60%) with *ad libitum* access to food and water.

Treatment design

C57BL/6J mice and ApoE^{-/-} mice were separated into two groups following a one-week adaptation period. C57BL/6J mice were fed a normal diet and served as the wild-type (WT) group, while ApoE^{-/-} mice were fed a high-fat diet for two months. Subsequently, the ApoE^{-/-} mice were allocated randomly into four groups: (1) ApoE^{-/-} group (ApoE^{-/-}, given distilled water); (2) AI low-dose group (ApoE^{-/-} + AI-L, 2 mg/kg/day); (3) AI medium-dose group (ApoE^{-/-} + AI-M, 4 mg/kg/day); and (4) AI high-dose group (ApoE^{-/-} + AI-H, 8 mg/kg/day). Starting at week 9, mice in the treatment groups received daily oral gavage of AI at the respective doses for 4 consecutive weeks. Behavioral testing was conducted for seven consecutive days following the final treatment.

Network pharmacology

Genes related to AD and atherosclerosis were retrieved from the DisGeNET database (<https://www.disgenet.org>) and the GeneCards database (<https://www.genecards.org>). After merging the datasets and removing duplicate entries, the common targets shared by the two diseases were identified using a Venn diagram generated on the Bioinformatics platform (<http://www.bioinformatics.com.cn>). To further clarify the relationships between the diseases and their corresponding targets, a disease-target network was constructed using Cytoscape 3.7.1 (<https://www.cytoscape.org/>). Subsequently, the network data were exported, and 27 key targets were selected according to their degree values. These targets were then imported

into the STRING database (<https://cn.string-db.org>), with the species limited to *Homo sapiens*, to construct a protein–protein interaction (PPI) network.

Molecular docking analyses

The RCSB PDB (<http://www.rcsb.org/>) provided the crystal structures of the atractylenolide I protein targets, which were then modified for target-ARG1 (PDB ID: 8e5m) and nNOS (PDB ID: 8bi9) using AutoDock Tools 1.5.6. These modifications included the elimination of ligands and water, hydrogenation, and amino acid optimization and repair. The files were subsequently saved in PDBQT format. In addition, the mechanical structure of small molecules was optimized with ChemBioDraw 3D, with the outcomes stored in MOL2 format which were then imported into AutoDock Tools 1.5.6, where all flexible bonds were set to be rotatable by default and saved in PDBQT format for docking. PyMOL was used to display the docking results, and AutoDock Vina 1.1.2 was used for the docking process.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analysis was performed by TGT MED (Shanghai, China) using a BIAcore S200 system to quantify the direct binding affinity between atractylenolide I and ARG1. ARG1 protein (Cusabio, Wuhan, China) was immobilized on a CM5 sensor chip via amine coupling using standard NHS/EDC chemistry. Atractylenolide I was serially diluted in PBST running buffer (0–100 μ M) and injected over the functionalized surface at 30 μ L/min. Binding kinetics were analyzed using BIAcore Evaluation Software with a steady-state affinity model to determine the equilibrium dissociation constant (K_D) values.

Morris water maze test

The platform was submerged one centimeter below the surface of the water maze pool, which was equally divided into four quadrants. On the first day of the experiment, following the treatment period, the mice were trained. Each mouse was given 1 min to find the platform and remained on it for 20 s. The training was conducted in three different quadrants for each mouse. On the final day, the mice were positioned at the starting point in the quadrant across the target platform after the platform was removed and relocated. Each mouse was allowed 1 min to search for the platform. A camera was used to track the swimming trajectory and record the time required to find the platform, and software was used to quantify the frequency and duration of crossings over the testing platform.

Serum biochemical assays

The serum levels of TG, T-CHO, LDL-C, and HDL-C were determined using biochemical kits (Nanjing Jiancheng) in accordance with the manufacturer's protocols. Briefly, appropriate amounts of serum and reaction reagents were mixed and incubated under the recommended conditions. The absorbance was then detected at the corresponding wavelength using a microplate reader (Thermo Fisher Scientific, Waltham, USA) and the serum lipid levels were calculated according to the standard procedure provided by the kit manufacturer.

^1H NMR spectroscopy of cerebral cortex tissue

Following thawing, 200 μ L of chloroform and 400 μ L of distilled

water were added to 100 mg of mouse cortical tissue that had been placed in a 1.5-mL EP tube. The samples were homogenized for 3 min in an ice bath. After the homogenate was centrifuged at 15,871 g and 4°C for 20 min, 500 μ L of the supernatant was transferred to a fresh 1.5-mL EP tube. With the aid of a vacuum concentrator, the sample was dried and the dehydrated samples were dissolved in 600 μ L of phosphate buffer solution to minimize changes in pH and then centrifuged again at 18,407 g and 4°C for 10 min. A total of 550 μ L of the supernatant was poured into a 5-mm NMR tube. The cortical samples were analyzed using a Bruker 600 MHz Avance III NMR instrument (Bruker BioSpin, Rheinstetten, Germany) at 25°C. The detection spectrum width was set to 7194.2 Hz, with 64 scans, an FID resolution of 0.188 Hz, a pulse duration of 9.95 s, a sampling time of 4.55 s, and a delay time of 1.0 s. There were 32,768 sampling points overall, with a 40.5-s sampling period. For basic data analysis, SIMCA-P 13.0 (Umetrics AB, Umeå, Sweden) was used for multivariate analysis of the NMR data, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). SIMCA-P 13.0 was also used to visually analyze the data, with the correlation coefficients R^2_X and Q^2_Y used to assess model quality. S-plots ($P < 0.05$) and VIP values ($VIP > 1$) were used to identify differential metabolites. For advanced data analysis, the identified differential metabolites were imported into MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca/>) for metabolic pathway analysis. In brief, metabolite identities were mapped to the *Mus musculus* pathway library via the built-in compound database. Pathway enrichment analysis and topology analysis were subsequently performed to identify metabolic pathways significantly associated with the observed alterations. Pathways were prioritized based on lower P values and higher pathway impact scores.

Histochemical analysis

After anesthesia, the mice were perfused transcardially with 0.05 M PBS, and some were fixed with 4% paraformaldehyde (PFA) for perfusion. The corresponding tissues were collected for preservation. Brain tissues were removed and fixed in 4% PFA solution. After dehydration, the tissues were embedded in paraffin. Paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol, and stained with Nissl staining solution (Beyotime, Shanghai, China) for 10 min. After a brief rinse in running water, the sections were dehydrated, cleared, and mounted with neutral balsam. Morphological features were observed under an ECLIPSE 80i microscope (Nikon, Tokyo, Japan).

Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) (Leagene, Beijing, China). The left lobe of the liver was cut lengthwise and fixed in 4% paraformaldehyde solution. After dehydration, the sample was immediately embedded in paraffin. The specimens were then sectioned into 4- μ m-thick slices and mounted on slides for H&E staining. The morphological characteristics of the liver tissue were examined under the ECLIPSE 80i microscope.

Oil Red O staining of the liver was performed as follows. The left lobe of the liver was dissected longitudinally, preserved in a 4% paraformaldehyde solution before being dehydrated in a 30% sucrose solution, and embedded on ice. After being cut into 15- μ m-thick slices, the samples were placed on slides. The sections were immersed in the Oil Red O dye solution (Nanjing Jiancheng) for

10 min, rinsed in warm distilled water (~37 °C) for 20 s, counterstained for 3 min, and rinsed again for 60 s. Finally, a water-based mounting medium was applied to seal the slides. The morphological characteristics of the liver tissue were examined under the ECLIPSE 80i microscope. Oil Red O staining of the aorta was performed as follows. After the aorta was removed with physiological saline, it was fixed in 4% paraformaldehyde for 48 h. The residual paraformaldehyde was removed by washing with PBS. The samples were then gradually dehydrated in gradient ethanol solutions, soaked in a clearing agent for transparency, and finally immersed in Oil Red O dye solution for 3 min.

Immunofluorescence staining

Immunofluorescence staining of the brain was performed as follows. Brain tissues were dehydrated, pelleted, and embedded in OCT compound. Coronal sections (10–15 µm) were prepared using a cryostat microtome, mounted on glass slides, air-dried for 15 min, and baked at 37°C for 10–20 min. The sections were blocked with 10% bovine serum albumin (BSA; Servicebio, Wuhan, China) in PBS for 30 min at room temperature, followed by incubation overnight at 4°C with primary antibodies: anti-ZO-1 (1:200) and anti-OCN (1:200). After being washed with PBS (3 × 5 min), the sections were incubated with the Alexa Fluor 488-conjugated goat anti-rabbit IgG (Servicebio) at room temperature for 50 min. The nuclei were counterstained with DAPI (Servicebio) at room temperature for 10 min, anti-fluorescence quenchers (Servicebio) were added when the plates were sealed, and the cells were immediately observed and photographed under an Eclipse C1 fluorescence microscope (Nikon).

Real-time PCR analysis

Total RNA extracted from mouse brain tissue was reverse-transcribed into cDNA. PrimeScript™ RT Master Mix Kit (Takara, Beijing, China) was used for reverse transcription. Quantitative PCR was carried out with a SYBR® Green Premix Pro Taq HS qPCR Kit (AG, Hangzhou, China) in a total volume of 20 µL. All tests were performed in triplicate. The qPCR conditions were as follows: predenaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s, followed by a dissociation stage. The primer sequences are listed in Table 1.

Western blot analysis

Brain tissue was collected for western blot analysis. RIPA lysis buffer (P0013C; Beyotime) containing 1× protease and phosphatase inhibitors was added to the tissue at a 1:9 tissue-to-lysate ratio. The samples were homogenized for 15 min at 4°C and lysed on ice for 30 min. The homogenate was centrifuged at 845 g and 4°C for 30 min to collect the supernatant. The protein concentration was quantified using a BCA protein assay kit (Thermo Fisher Scientific), with BSA as the standard. Each sample in equal parts was subjected to 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore, Billerica, USA). After being blocked in 5% BSA, the membranes were incubated at 4°C overnight with primary antibodies. After incubation, the membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBST; Servicebio) solution (10 min per wash) and incubated with HRP-linked anti-rabbit IgG antibody (CST) at room temperature for 2 h. Finally, an enhanced chemiluminescence device (Bio-Rad Labora-

Table 1. Sequences of primers used for qPCR

Gene	Primer sequence	Length (bp)
<i>ARG1</i>	F 5'-GTAGACCCTGGGGAACACTAT-3'	21
	R 5'-ATCACCTTGCCAATCCCCAG-3'	20
<i>nNOS</i>	F 5'-CGCCTCACTTATGTGGCAGA-3'	20
	R 5'-GATGGTCGATCGGCTGGATT-3'	20
<i>iNOS</i>	F 5'-TCTAGTGAAGCAAAGCCCAACA-3'	22
	R 5'-CCTCACATACTGTGGACGGG-3'	20
<i>ABCA1</i>	F 5'-CCTGAAGCTGACCAAGGTGTG-3'	21
	R 5'-TGATTCCATACTGGCTCGGG-3'	20
<i>LXR</i>	F 5'-AGCGGCAAGAAGAGGAAC-3'	18
	R 5'-TCAGTAAAGTGGGCAAAGC-3'	19
<i>BAX</i>	F 5'-AAACTGGTGCTCAAGGCC-3'	19
	R 5'-GGTCCCAAGTAGGAGAGGA-3'	20
<i>BCL-2</i>	F 5'-GGGGTCATGTGTGTGGAGAG-3'	20
	R 5'-GTTCCACAAAGGCATCCCAG-3'	20
<i>α-Tubulin</i>	F 5'-CCTAAACAGTTGATAGGCCAAA-3'	23
	R 5'-CTCGCCTTCCACAGAATCCA-3'	20

F, forward; R, reverse.

tories, Hercules, USA) was used to detect the signals. Densities of immunoreactive bands were analyzed using ImageJ software, with β-Actin serving as the loading control.

Statistical analysis

All the statistical analyses were performed with GraphPad Prism software, version 9.5, with the data presented as the mean ± standard error of the mean (SEM). Changes in mouse body weight were analyzed using repeated-measures ANOVA with a general linear model. On the basis of the results of the sphericity test, multivariate ANOVA was applied to determine whether body weight changes differed significantly between groups. Additionally, one-way ANOVA was used to assess differences among groups for normally distributed data with homogeneity of variance. A *P* value of < 0.05 was considered to indicate statistically significance.

Results

Network pharmacology-based analysis

We first screened 2060 genes related to AD using the GeneCards database and 544 genes using the DisGeNET database and identified overlapping 241 genes between the two datasets (Figure 1A). Similarly, we identified 1347 and 2044 atherosclerosis-related genes, with 838 genes shared between the two datasets (Figure 1B). Next, we compared the genes associated with both diseases and identified 107 common regulatory genes (Figure 1C,D). The STRING11.0 database was populated with these 107 overlapping targets, with a minimum necessary interaction score of > 0.9. This analysis revealed 52 targets, which were used to construct a PPI network using Cytoscape 3.7.1 (Figure 1E). The network comprised 52 nodes and 760 edges, with APOE being the node with the highest degree.

As shown in Figure 1F and Table 2, KEGG pathway enrichment analysis revealed that the core genes were enriched primarily in pathways related to lipid metabolism and atherosclerosis, cholesterol metabolism, malaria, fluid shear stress and atherosclerosis,

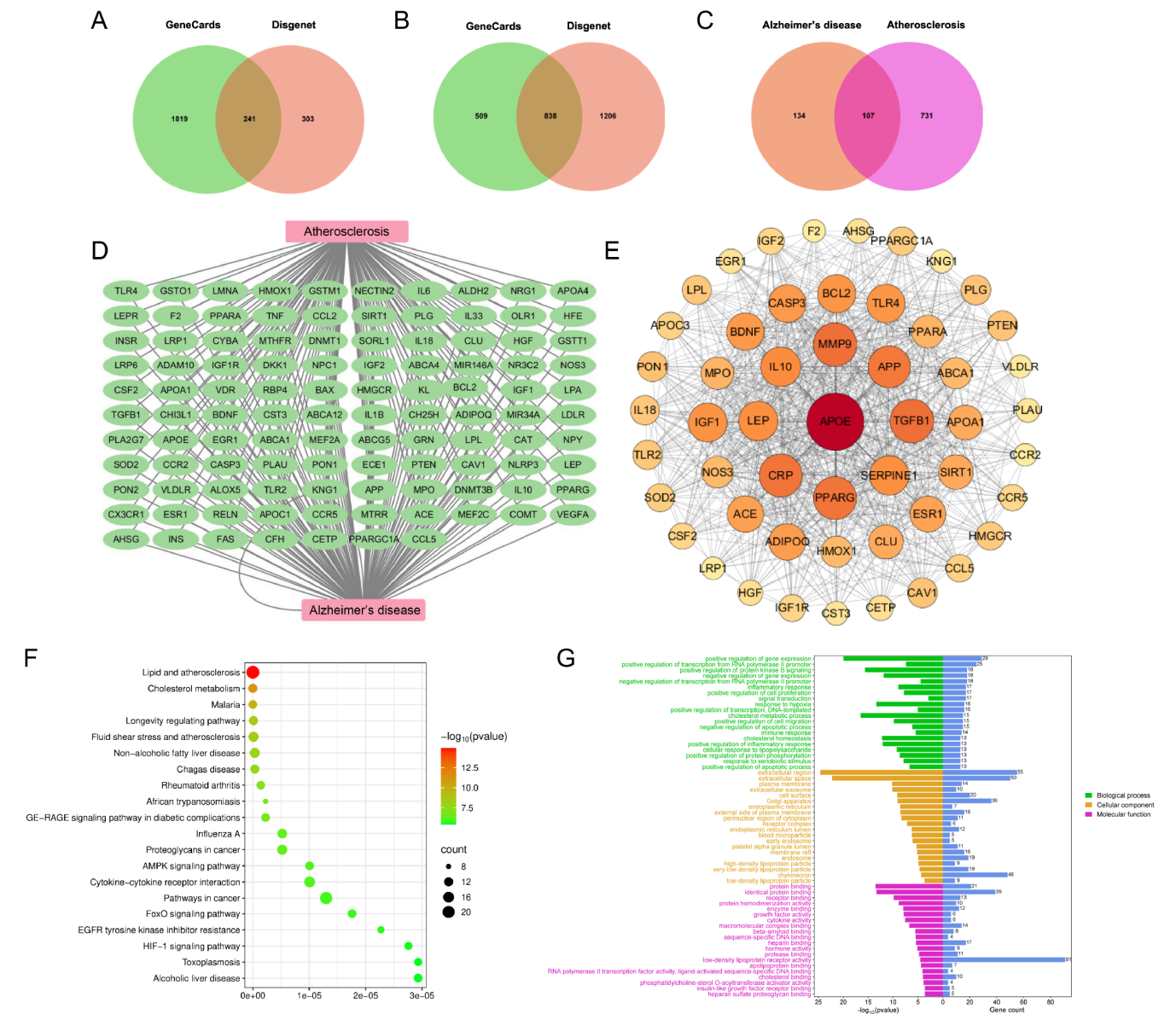


Figure 1. Network pharmacology-based analysis (A) Venn diagram showing the targets linked to AD. (B) Venn diagram showing the targets linked to atherosclerosis. (C) Venn diagram of AD and atherosclerosis targets. (D) AD-atherosclerosis target interaction network. (E) Protein-protein interaction network of proteins corresponding to the potential hub genes. Circle indicate a gene, the greater the degree, the larger the circle, and the darker the color (red). (F) KEGG enrichment analysis: Top 20 KEGG pathways associated with AD and atherosclerosis. (G) GO-BP enrichment analysis: Biological process, Cellular component, and Molecular function.

Table 2. Top 8 KEGG pathways associated with Alzheimer's disease and atherosclerosis

Pathway	P value	Genes	Count
Lipid and atherosclerosis	$P < 0.001$	ABCA1, NOS3, IL18, APOA1, APOA4, CYBA, VLDLR, SOD2, TNF, IL6, IL1B, CASP3, CCL5, BCL2, FAS, BAX, CCL2, OLR1, NLRP3, PPARG, LDLR, TLR4, TLR2	23
Cholesterol metabolism	$P < 0.001$	ABCA1, CETP, ABCG5, NPC1, LRP1, APOC1, LPL, APOA1, APOA4, APOE, LPA, LDLR	12
Malaria	$P < 0.001$	IL10, IL6, TGFB1, LRP1, IL1B, HGF, IL18, CCL2, TNF, TLR4, TLR2	11
Longevity regulating pathway	$P < 0.001$	KL, INSR, ADIPOQ, CAT, BAX, PPARG, IGF1, SOD2, SIRT1, PPARGC1A, IGF1R, INS	12
Fluid shear stress and atherosclerosis	$P < 0.001$	MEF2A, MEF2C, GSTM1, GSTO1, NOS3, CAV1, GSTT1, CYBA, TNF, VEGFA, IL1B, BCL2, CCL2, HMOX1	14
Non-alcoholic fatty liver disease	$P < 0.001$	TGFB1, INSR, ADIPOQ, TNF, INS, IL6, IL1B, CASP3, LEP, LEPR, FAS, BAX, PPARG, PPARG	14
Chagas disease	$P < 0.001$	IL10, IL6, TGFB1, ACE, CCL5, IL1B, FAS, CCL2, TNF, TLR4, KNG1, TLR2	12

longevity, Chagas disease, and non-alcoholic fatty liver disease. Notably, several lipid-related genes, especially *APOE* and *ABCA1*, were repeatedly identified across multiple pathways, suggesting their central roles in the pathological overlap between AD and atherosclerosis. Similarly, as shown in Figure 1G, GO-BP enrichment analysis revealed that the core genes played substantial roles in the positive regulation of gene expression, positive regulation of transcription from the RNA polymerase II promoter, positive regulation of protein kinase B signaling, negative regulation of gene expression, negative regulation of transcription from the RNA polymerase II promoter, inflammatory response, positive regulation of cell proliferation, and signal transduction.

These results indicate that *APOE* is a key gene that links atherosclerosis and AD, both of which are closely associated with lipid and cholesterol metabolism.

AI ameliorates AD pathology and restores cognitive function in ApoE^{-/-} mice

To further explore the specific relationship between AD and atherosclerosis and to investigate potential therapeutic strategies, we conducted *in vivo* experiments using ApoE^{-/-} mice fed an HFD. Behavioral tests were then conducted to evaluate the protective

effects of AI on cognitive performance in ApoE^{-/-} mice. Compared with WT mice, ApoE^{-/-} mice showed marked impairments in spatial learning and memory, as evidenced by fewer platform crossings, reduced time and distance in the target quadrant, and altered swimming behavior during the Morris water maze test (Figure 2A–F). In the probe trial, AI-treated mice traversed the platform area multiple times and spent a significant amount of time in the target quadrant in comparison to ApoE^{-/-} mice after the hidden platform was removed (Figure 2A,B). Compared to ApoE^{-/-} mice, AI-treated mice exhibited significantly shorter escape latencies during the 5-day training phase (Figure 2C), indicating spatial learning and memory recovery. In addition, compared with ApoE^{-/-} mice, AI-treated mice showed increased time spent in the target quadrant and greater distance traveled in the target quadrant, accompanied by partial restoration of average swimming velocity (Figure 2D–F), suggesting that AI improved both their cognitive and motor abilities. These results indicate that AI can reverse cognitive decline in ApoE^{-/-} mice.

Given the crucial role of ApoE in both atherosclerosis and AD, we subsequently investigated the effects of ApoE deficiency on the cellular architecture and structural integrity of the brain by Nissl staining. As shown in Figure 2G, pronounced morphologic

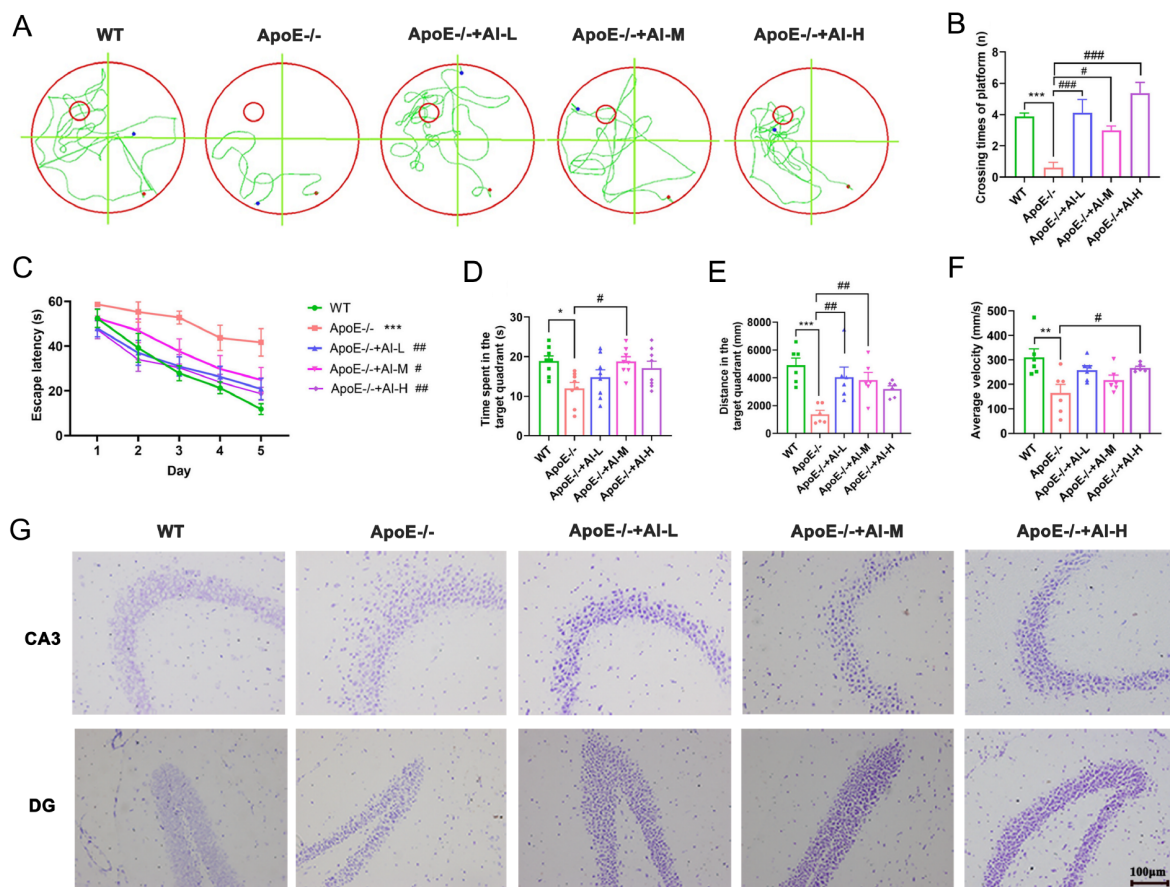


Figure 2. AI reverses cognitive impairment in ApoE^{-/-} mice (A) Representative swimming trajectories of the mice during the Morris water maze test ($n = 8$). (B) The number of target platform crossings during the probe test. (C) Escape latency training for five consecutive days in ApoE^{-/-} mice. (D) Time spent in the target quadrant. (E) Distance actively travelled in the target quadrant. (F) Average swimming velocity of the mice. (G) Representative microphotographs of the CA3 and DG areas of hippocampal sections from the experimental groups stained with Nissl staining. Nissl bodies were dyed deep blue, and the cell nuclei were lightly stained. Scale bar = 100 μm . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs WT. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs ApoE^{-/-}.

alterations and various cellular anomalies were observed across the different groups. The ApoE^{-/-} group exhibited aberrant cellular morphology, with disrupted tissue structure and irregular cell distribution, whereas the WT group exhibited typical cellular morphology with no discernible changes. There was a gradual degree of morphological restoration and a tighter and more organized cell arrangement after intervention with AI, suggesting that AI mitigated the damage to the neurons. ApoE deficiency is strongly associated with blood-brain barrier (BBB) leakage [27]. The BBB is a critical line of defense against harmful external stimuli and is composed of endothelial and epithelial cells joined by tight junction proteins and supported by multiple other structures. To investigate the effect of ApoE deficiency on BBB integrity, changes in the expression of the tight junction proteins zonula occludens-1 (ZO-1) and occludin (OCLN) were detected by immunofluorescence staining (Figure 3A,B) and western blot analysis (Figure 3C). Compared with the WT group, ApoE^{-/-} group showed decreased expression of ZO-1 and OCLN in the cortex and hippocampus, suggesting disruption of BBB integrity. AI treatment upregulated the expression of these tight junction proteins, especially in the middle-dose group, indicating that AI ameliorated BBB damage and helped preserve BBB integrity. Notably, BBB leakage can lead to the accumulation of agents such as lipids and A β in the brain, promoting amyloid plaque formation [28]. Consistent with previous findings, ApoE^{-/-} mice showed significantly higher brain A β levels than WT mice, suggesting pronounced A β accumulation in the ApoE^{-/-} mice (Figure 3D). After AI treatment, brain A β levels were significantly decreased in the AI-L, AI-M, and AI-H groups relative to the ApoE^{-/-} group, with the reduction appearing more pronounced in the medium- and high-dose groups, suggesting that AI effectively alleviated A β deposition in ApoE^{-/-} mice.

AI inhibits neuronal apoptosis in ApoE^{-/-} mice

Extensive neuronal apoptosis is a major factor contributing to the development and progression of AD. Key regulators of the mitochondrial apoptosis pathway include *Bcl-2*, *Bax*, Caspase-3, and Caspase-9. Abnormal lipid metabolism promotes apoptosis by inhibiting *Bcl-2* activity and increasing *Bax* levels, thereby triggering Caspase-3-dependent apoptosis. To assess apoptosis-related factors in the hippocampus, we performed western blot analyses of *Bcl-2*, *Bax* (Figure 4A,C), Caspase-3, and Caspase-9 (Figure 4B,E), and further measured the mRNA expression of *Bcl-2* and *Bax* by qPCR, followed by calculation of the *Bcl-2*/*Bax* ratio (Figure 4D). Western blot analysis showed that, compared with WT mice, ApoE^{-/-} mice exhibited decreased *Bcl-2* expression and increased the expression levels of *Bax*, Caspase-3 and Caspase-9, indicating enhanced neuronal apoptosis. Consistent with the findings of previous studies [29,30], AI treatment decreased *Bax*, Caspase-3, and Caspase-9 expression while increasing *Bcl-2* expression, suggesting that AI suppressed neuronal apoptosis, as indicated by the western blot results. Moreover, qPCR analysis showed that the *Bcl-2*/*Bax* mRNA ratio was significantly reduced in ApoE^{-/-} mice compared with WT mice. AI intervention increased the *Bcl-2*/*Bax* mRNA ratio, with significant improvement observed in the medium- and high-dose groups, indicating that AI may suppress apoptosis at the transcriptional level. These findings indicate that AI ameliorated hippocampal cytoarchitectural disruption and neuro-behavioral deficits, thereby improving cognitive function in HFD-treated ApoE^{-/-} mice. Moreover, AI abrogated HFD-induced amyloid

plaque formation in the hippocampus, confirming its protective effects against AD through preservation of BBB integrity and inhibition of neuronal apoptosis.

AI ameliorates lipid metabolism disorders in ApoE^{-/-} mice fed an HFD

We next examined the effects of an HFD on metabolic parameters in ApoE^{-/-} mice. As shown in Figure 5A, body weight progressively increased in ApoE^{-/-} mice relative to WT mice from week 4 of HFD feeding, and this difference was sustained throughout the 12-week study period, ultimately resulting in obesity. In addition, serum biochemical assays revealed that the ApoE^{-/-} mice fed an HFD exhibited severe dyslipidemia, characterized by increased levels of TG, T-CHO, and LDL-C, along with reduced HDL-C levels. Notably, these effects were markedly alleviated by AI intervention (Figure 5C). Moreover, HFD consumption caused marked hepatic lipid accumulation in ApoE^{-/-} mice. Compared with the WT mice, the ApoE^{-/-} mice exhibited significantly increased liver levels of TG, T-CHO, and LDL-C and decreased HDL-C levels (Figure 5D). These results suggest a substantial increase in free cholesterol levels during the early stages of disease development in ApoE^{-/-} mice fed an HFD.

Histological staining revealed characteristics of an HFD-induced hepatic steatosis, which was mitigated by AI intervention. H&E staining and Oil Red O staining demonstrated that liver lipid accumulation and inflammatory infiltration (Figure 5E, green arrows) in HFD-fed mice were notably ameliorated by AI treatment, as supported by hepatic and serum lipid biochemical results (Figure 5C,D). In addition, plaque development is a key phenotypical hallmark of atherosclerosis. Compared with WT mice, ApoE^{-/-} mice fed an HFD presented more red-stained plaques in aortic regions; conversely, AI treatment markedly reduced aortic wall plaque deposition in ApoE^{-/-} mice to levels comparable to those in WT mice (Figure 5B). Given that dysregulated cholesterol homeostasis in the brain contributes to the clinical manifestations of AD and that cholesterol deposition is frequently observed in AD patients, we further assessed lipid metabolism in the brain. Brain lipid profiling revealed that ApoE^{-/-} mice exhibited marked disturbances in lipid metabolism; however, AI treatment effectively restored the levels of TG, T-CHO, and LDL-C to values comparable to those of WT mice (Figure 5F). Collectively, our data demonstrated that AI provides substantial protection against HFD-induced dyslipidemia and lipid overload in ApoE^{-/-} mice, as evidenced by improvements in peripheral and central lipid profiles, attenuation of hepatic steatosis, and reduction of aortic plaque deposition.

AI promotes cholesterol efflux and reduces M1 macrophage polarization

Disrupted brain cholesterol homeostasis is widely recognized as a key contributor to AD pathogenesis [31,32]. ABCA1, a critical regulator of reverse cholesterol transport, has been implicated in AD development because it mediates the transfer of intracellular cholesterol to apolipoprotein AI (APOAI) and high-density lipoprotein [33]. As ABCA1 is a direct transcriptional target of LXR, we further examined the effects of AI on the LXR/ABCA1 signaling axis in the hippocampus of ApoE^{-/-} mice. Compared with WT mice, ApoE^{-/-} mice showed significantly reduced protein expression of LXR and ABCA1 in the hippocampus (Figure 6A) and lower mRNA expression of both genes (Figure 6B,C). AI treatment significantly upregulated both LXR and ABCA1 at the protein and mRNA levels,

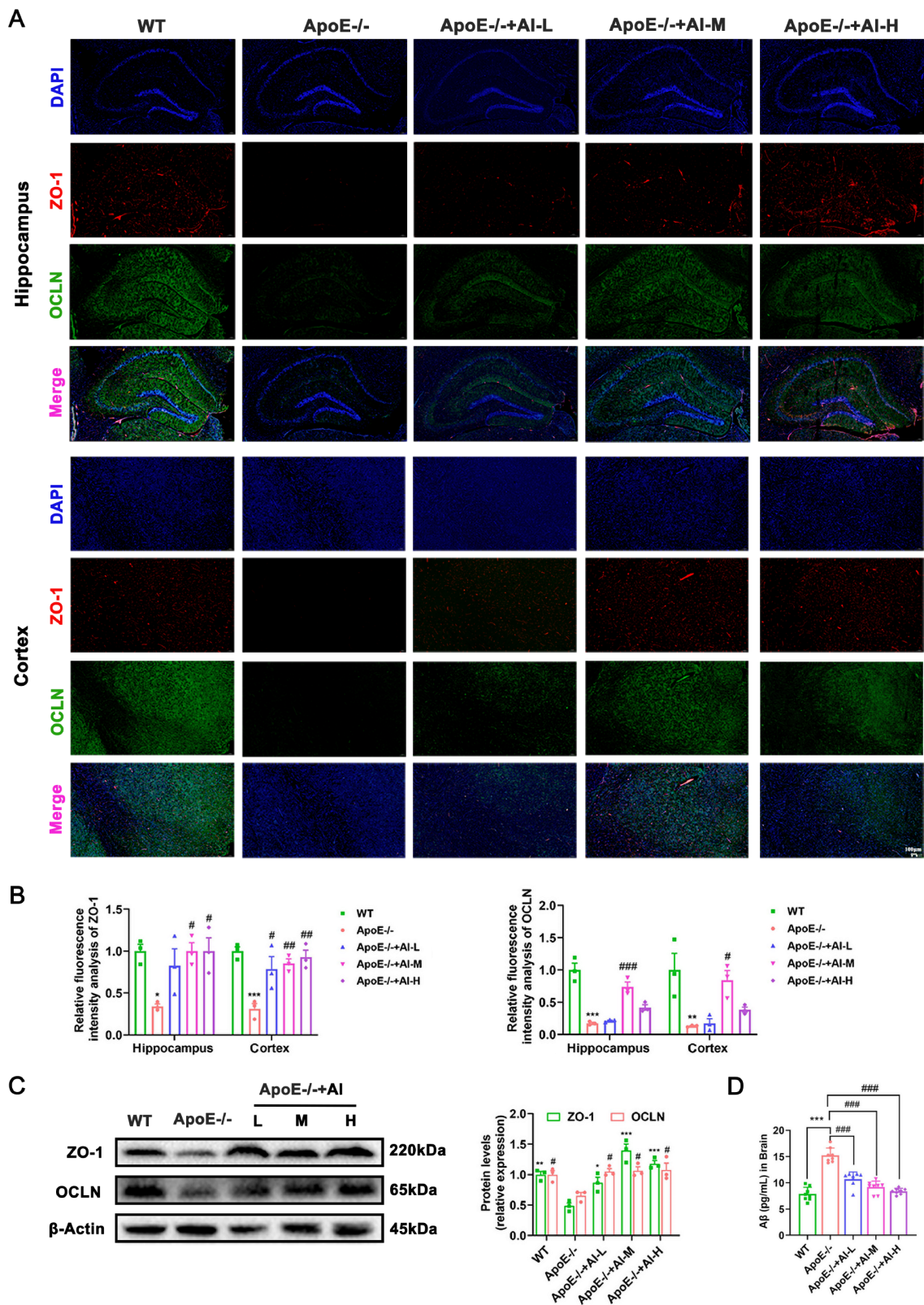


Figure 3. AI mitigates BBB damage through restoration of tight junction-related proteins of ApoE^{-/-} mice (A) Representative immunofluorescence images of ZO-1 and OCLN in cortex and hippocampal sections from the experimental groups. Scale bar = 100 μm. (B) Relative fluorescence intensity analysis of ZO-1 and OCLN in cortex and hippocampus. (C) Western blot analysis showing the ZO-1 and OCLN levels in the hippocampus of the indicated groups of mice. Quantitative WB analysis of the ZO-1 and OCLN normalized to β-Actin levels. (D) Aβ levels in the hippocampus. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs WT. #*P* < 0.05, ###*P* < 0.01, ####*P* < 0.001 vs ApoE^{-/-}.

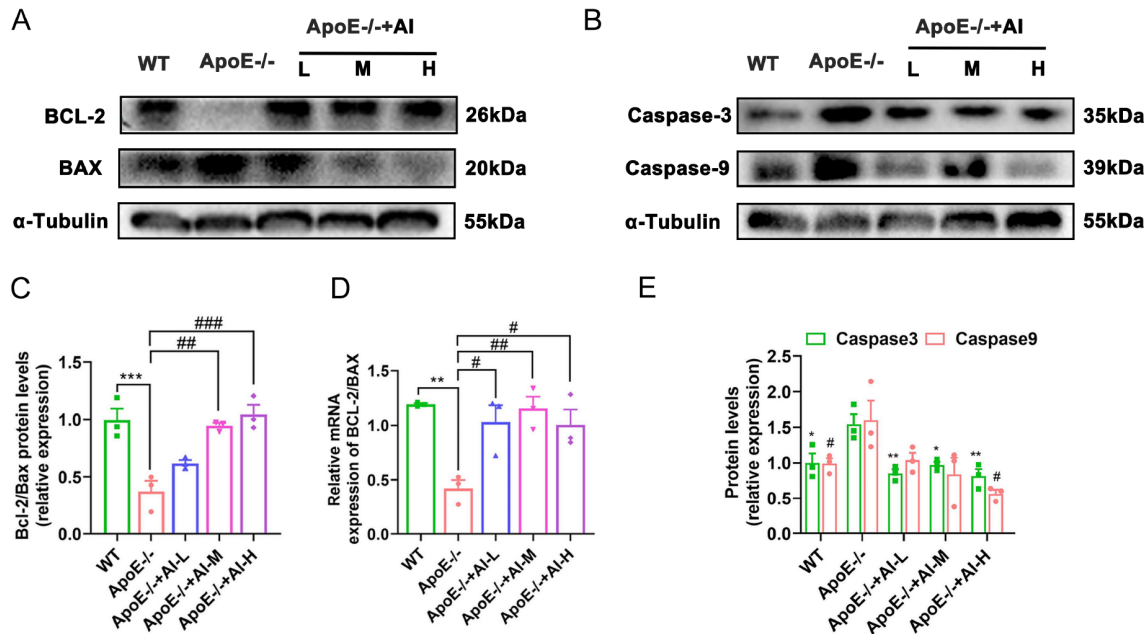


Figure 4. AI inhibits neuronal apoptosis in *ApoE*^{-/-} mice (A,B) Western blot analysis showing the Bcl-2, Bax, Caspase-3 and Caspase-9 levels in the hippocampus of the indicated groups of mice. (C) Quantitative WB analysis of the Bcl-2 and Bax levels normalized to α -Tubulin levels. (D) qPCR analysis showing the Bax and Bcl-2 expression in the hippocampus. (E) Quantitative WB analysis of the Caspase-3 and Caspase-9 levels normalized to α -Tubulin levels (* P < 0.05, ** P < 0.01 vs *ApoE*^{-/-} for Caspase-3; * P < 0.05, ** P < 0.01 vs *ApoE*^{-/-} for Caspase-9). * P < 0.05, ** P < 0.01, *** P < 0.001 vs WT. # P < 0.05, ## P < 0.01, ### P < 0.001 vs *ApoE*^{-/-}.

suggesting a potential enhancement of cholesterol efflux signaling in the hippocampus. Thus, AI may ameliorate lipid metabolism abnormalities in *ApoE*^{-/-} mice, possibly through activation of the LXR/ABCA1 axis.

Given the observed lipid accumulation in HFD-fed *ApoE*^{-/-} mice, we next examined neuroinflammatory changes in the hippocampus. We measured the protein levels of M1 macrophage markers and phenotype-specific cytokines in the hippocampus. Our results indicated that, compared with WT mice, *ApoE*^{-/-} mice exhibited increased iNOS mRNA expression (Figure 6D) along with elevated protein expression of IL-1 β and iNOS (Figure 6E). In addition, the levels of TNF- α and IL-6 were markedly elevated in *ApoE*^{-/-} mice (Figure 6F,G). After AI treatment, IL-1 β and iNOS expression showed a downward trend, and TNF- α and IL-6 levels were significantly reduced, particularly in the medium- and high-dose groups. Activated cerebral macrophages release proinflammatory cytokines, such as TNF- α , IL-6, and other toxic molecules, driving BBB disruption and neuronal apoptosis [34]. Specifically, iNOS-derived nitric oxide induces mitochondrial damage and neuronal apoptosis [35], while IL-1 β exacerbates BBB dysfunction and promotes apoptosis [36]. These data collectively suggest that AI inhibited M1 macrophage polarization, as reflected by decreased expression of all measured M1-associated markers (iNOS, IL-1 β , TNF- α , and IL-6).

AI regulates arginine biosynthesis-related metabolites via the ARG1-nNOS axis

Untargeted metabolomics was performed to assess the metabolic effects of AI in *ApoE*^{-/-} mice. A representative ¹H-NMR spectrum from the WT mice (Figure 7A) displayed high signal intensity and peak capacity, providing a robust basis for metabolite identification. Characteristic peaks were matched to reference standards using

NMR databases (HMDB and BMRB) to identify metabolites (Table 3). Multivariate analysis (SIMCA-P 13.0) of the NMR data established principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA) models. These models revealed clear separation between the WT and *ApoE*^{-/-} groups, as well as between the AI-treated and *ApoE*^{-/-} groups (Figure 7B), confirming significant metabolic alterations in HFD-fed mice. Differential metabolite screening based on OPLS-DA S-plots and VIP values revealed eight dysregulated metabolites in AI-treated mice vs *ApoE*^{-/-} mice (Figure 7C-E). MetaboAnalyst 5.0 was used to identify and predict candidate biomarkers and key metabolic pathways (Figure 7G). The results indicated that AI primarily affected arginine biosynthesis, glycine, serine, and threonine metabolism, and even pyrimidine and purine metabolism, with arginine biosynthesis being the most significantly altered pathway. Additionally, notable alterations in the concentrations of vital metabolites such as betaine and malic acid were detected. Compared with WT mice, *ApoE*^{-/-} mice showed increased levels of betaine and malic acid, whereas AI treatment elevated these elevations (Figure 7F). Consistent with the reported neuroprotective role of betaine [37,38], the AI-associated increase in betaine accumulation may enhance neuroprotection through inhibition of hippocampal inflammation and neuronal apoptosis. Notably, urea levels were also increased in *ApoE*^{-/-} mice relative to WT mice and were significantly decreased after AI treatment, suggesting that AI may ameliorate urea-cycle-related metabolic disturbances associated with AD [39].

ARG1 is a key factor in urea production, and is distinctly upregulated in areas of pronounced A β pathology in AD mice [40], making it a potential therapeutic target for AD. We therefore determined whether AI treatment affects the urea cycle by investigating its effect on ARG1 expression. Compared with WT

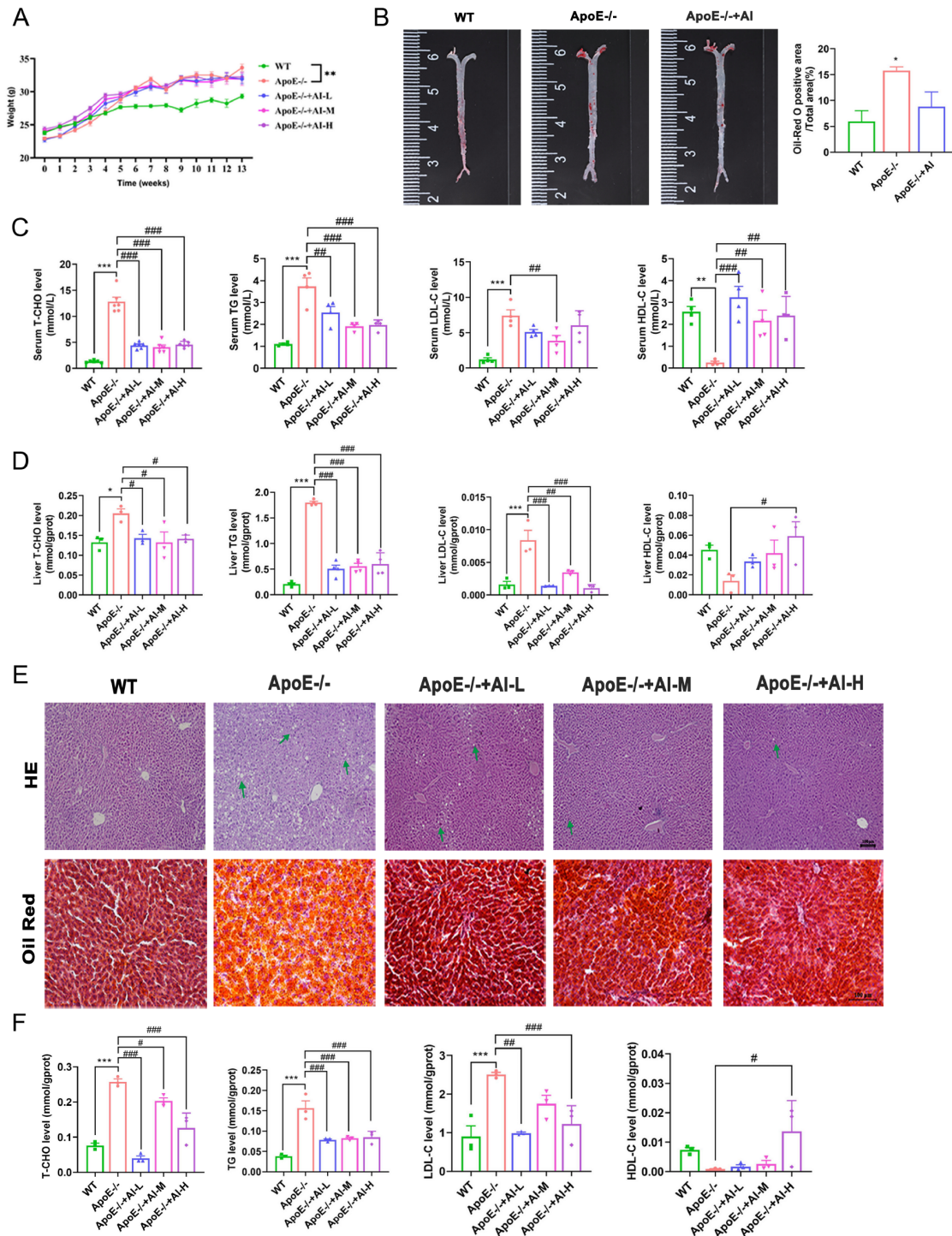


Figure 5. AI ameliorates lipid metabolism disorders in ApoE^{-/-} mice fed an HFD (A) Body weights of the mice over 0–13 weeks; Data are presented as the mean $\pm$ SEM. (B) Gross Oil Red O staining was used to evaluate the location and extent of mouse aortic lesions. (C) Serum lipid content of T-CHO, TG, LDL-C, and HDL-C. (D) Hepatic lipid content: T-CHO; TG; LDL-C; HDL-C. (E) Morphological analysis. Upper: H&E staining of liver tissues from ApoE^{-/-} mice; lower: Oil red O staining was used to identify lipid deposition in the liver. Scale bar = 100 μ m. (F) Brain lipid content of T-CHO, TG, LDL-C, and HDL-C. T-CHO, total cholesterol; TG, triglycerides; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol. * P < 0.05, ** P < 0.01, *** P < 0.001 vs WT. # P < 0.05, ## P < 0.01, ### P < 0.001 vs ApoE^{-/-}.

mice, ApoE^{-/-} mice exhibited increased ARG1 expression and decreased nNOS expression at both the protein and mRNA levels (Figure 7J,K). AI treatment partially reversed these changes, as evidenced by reduced ARG1 expression and increased nNOS

expression. nNOS, which is functionally connected to ARG1 through shared arginine metabolism, regulates central nervous system functions and is associated with arginine biosynthesis. nNOS concentration was significantly reduced in ApoE^{-/-} mice

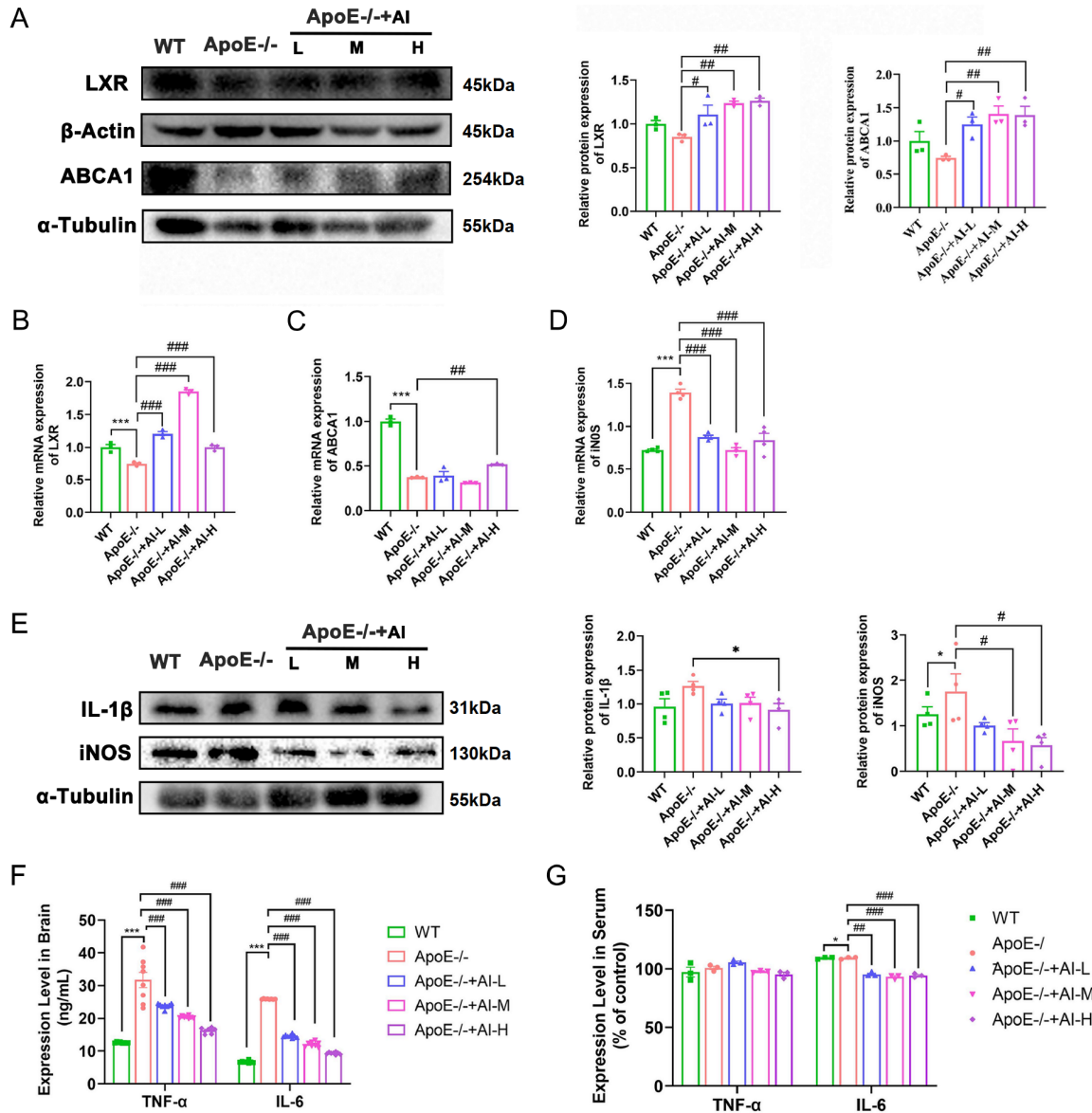


Figure 6. AI promotes cholesterol efflux and reduces M1 macrophage polarization (A) The expression of LXR and ABCA1 protein from brain tissue was investigated by western blot analysis. (B–D) The mRNA level of lipid efflux and macrophage polarization markers in brain tissue. (E) The expression of IL-1β and iNOS protein in brain tissue was investigated by western blot analysis. (F,G) The expression of proinflammatory factor in brain tissue (F) and serum (G) was examined by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs WT. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs ApoE^{-/-}.

compared with WT mice (Figure 7L). Compared with the ApoE^{-/-} group, AI dose-dependently increased the nNOS concentration in all treatment groups. Molecular docking simulations were employed to characterize the binding modes of AI with ARG1 and nNOS (Figure 7H,I), which revealed preferential binding affinity of AI for ARG1, as evidenced by its relatively stable conformation. Further SPR analysis revealed a K_D of 17.49 μ M for the AI-ARG1 interaction (Figure 7M). Collectively, these data suggest that AI is a selective modulator of ARG1, potentially regulating urea cycle dysregulation in the pathogenesis of AD.

Discussion

Emerging epidemiological evidence links atherosclerosis to accelerated AD pathology in middle-aged individuals [41], and our integrated database analysis revealed that ApoE-specific pathology

is a mechanistic link between these two conditions. Given the dual role of ApoE in A β clearance and lipid transport [42], it serves as a critical molecular bridge between AD and atherosclerosis. HFD-fed ApoE^{-/-} mice are well-established for recapitulating both atherosclerotic and neurodegenerative features [43], and our experiment confirmed three neurovascular pathologies: spatial memory deficits (prolonged latency in the Morris water maze test), BBB disruption (reduced expression of ZO-1 and OCLN), and A β overproduction. Maintenance of neural integrity has been shown to improve memory and cognitive function [44], which is critically dependent on BBB integrity. Notably, OCLN, the first transmembrane protein found in tight junctions, has been extensively studied for its role in maintaining BBB integrity [45]. Moreover, ZO-1, located at the presynaptic membrane, is involved in endocytosis, a crucial process in the release and control of neurotransmitters, thus impacting BBB

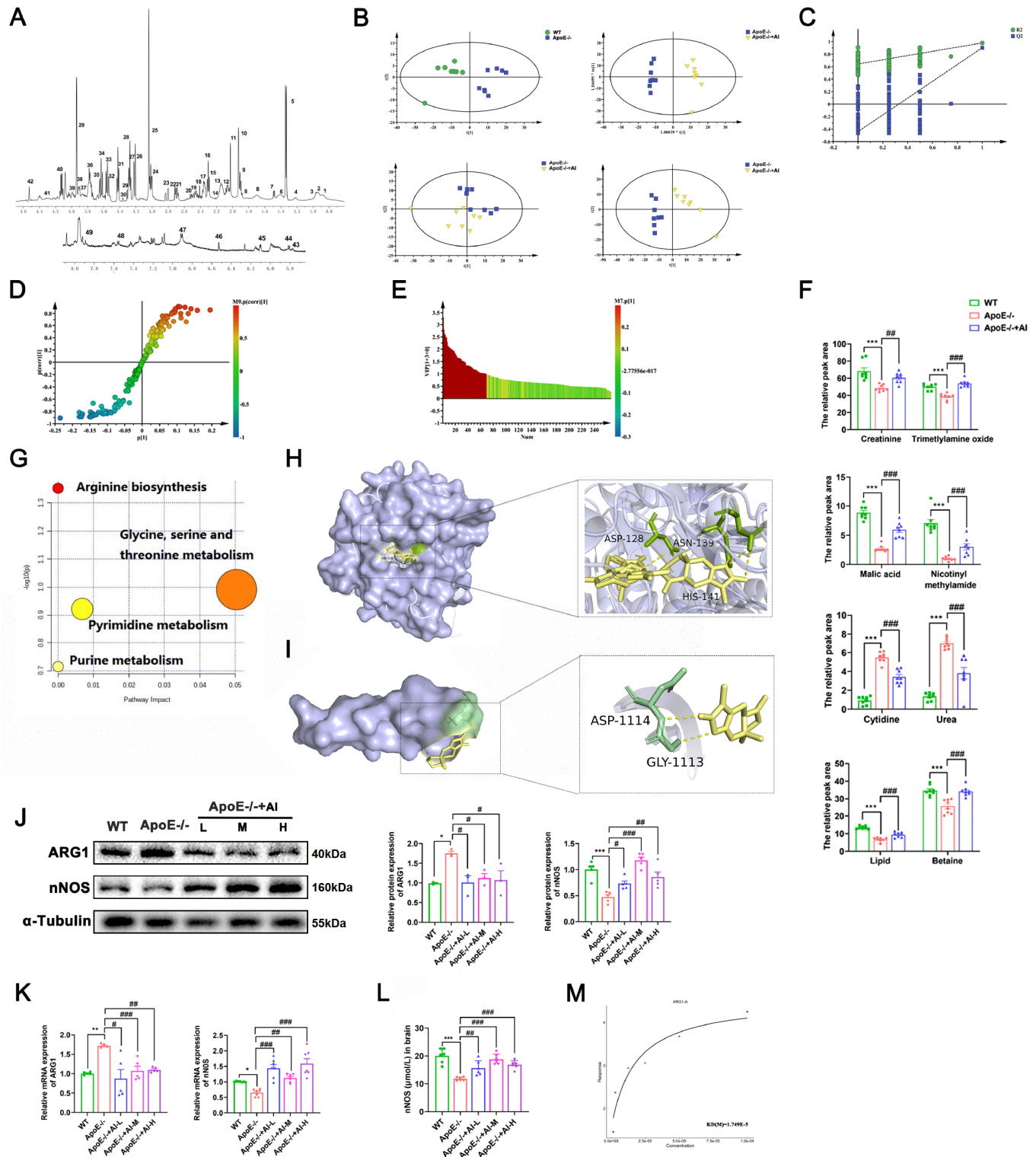


Figure 7. AI regulates arginine biosynthesis-related metabolites via the ARG1-nNOS axis (A) Representative ^1H -NMR spectra of WT mice. Numbers 1–49 represent different metabolites (Table 3). (B) PCA scatter plot (WT vs ApoE $^{-/-}$); PCA scatter plot (AI vs ApoE $^{-/-}$); PLS-DA scatter plot; OPLS-DA scatter plot. (C) PLS-DA cross-validation plot. (D) OPLS-DA S-plot. (E) OPLS-DA VIP values. (F) Comparison of the relative peak areas of different metabolites in each group: betaine; creatinine; lactate; lipids; malic acid; N-methyl nicotinamide; trimethylamine oxide; and urea. (G) Enrichment analysis of metabolic pathways linked to differentially abundant metabolites. The dimensions of the circle signify the strength of the correlation. The larger the circle, the stronger the correlation. (H,I) Simulation of the binding of AI with ARG1 (H) and nNOS (I) using molecular docking technology. (J) Relative protein expression levels of ARG1 and nNOS in the brain. (K) Relative mRNA expression of ARG1 and nNOS in the brain. (L) nNOS active concentration in the brain. (M) SPR analysis of AI binding to ARG1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs WT. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs ApoE $^{-/-}$.

Table 3. ¹H-NMR spectra of major metabolites of the brain

No.	Metabolite	δ ¹ H (multiplicity)
1	Lipid	0.87(m), 1.29(m), 1.58(m), 2.77(m)
2	Isoleucine	0.94(t, 7.4 Hz), 1.01(d, 7.0 Hz), 1.20(d, 6Hz)
3	Leucine	0.96(t, 6.3 Hz), 1.72(m)
4	Isobutyric acid	1.07(d, 7.5 Hz)
5	Lactate	1.33(d, 6.9 Hz), 4.12(q, 6.9 Hz)
6	2-Hydroxyisobutyrate	1.39s
7	Alanine	1.49(d, 7.3 Hz)
8	Lysine	1.72(m), 1.91(m), 3.01(m), 3.76(t)
9	Acetic acid	1.92s
10	Acetate	1.93s
11	N-acetyl-glycoprotein	2.04(s)
12	Glutamic acid	2.06(m), 2.14(m), 2.36(m)
13	Glutamine	2.15(m), 2.44(m)
14	Acetone	2.23(s)
15	Ethylacetoacetate	2.30(s)
16	Pyruvate	2.35(s)
17	Methionine	2.36(m), 2.06(m), 3.78(m), 2.16(m)
18	Succinic acid	2.41(s)
19	α-Ketoglutaric acid	2.44(t), 3.01(t)
20	Citrate	2.52d, 2.69d
21	Aspartic acid	2.69(d, 7.2 Hz), 2.81(d, 6 Hz)
22	Dimethylamine	2.72(s)
23	Trimethylamine	2.86(s)
24	Sarcosine	3.03(s), 3.93(s)
25	Creatinine	3.04(s), 4.05(s)
26	Phosphatidylcholine	3.21(s)
27	Choline glycerophosphate	3.22(s)
28	Trimethylamine oxide	3.26(s)
29	Betaine	3.27(t), 3.90(s)
30	Trimethylammonium oxide	3.35(s), 3.25(s)
31	Glucose	3.43(m), 3.47(m), 3.51(m), 3.87(m)
32	Glycine	3.55(s)
33	Glutamine	3.56(m), 2.71(m)
34	Inositol	3.63(s)
35	Inositol	3.65(dd, 7.2 Hz)
36	Guanidine acetic acid	3.80(s)
37	Glycerol	3.84(m)
38	3-Hydroxypropionic acid	3.85(dd, 7.2 Hz)
39	Phenylalanine	3.99(dd, 6.0 Hz)
40	D-3-Hydroxybutyric acid	4.15(t)
41	Malic acid	4.31(dd, 7.2 Hz)
42	N-methylnicotinamide	4.51(s), 8.18(t)
43	Urea	5.81brs
44	Uracil	5.81(d, 6.0 Hz)
45	Cytidine	6.07(d, 7.4 Hz)
46	Fumaric acid	6.53(s)
47	Tyrosine	6.89(d, 7.2 Hz)
48	Hippuric acid	7.56t, 7.49t
49	Xanthine	7.90s

function. Aβ, a known risk factor for both AD and atherosclerosis, colocalizes with cholesterol deposits in AD plaques [46], and similar results were observed in our study for increased brain Aβ production in ApoE^{-/-} mice. These findings collectively validate the utility and reliability of the ApoE^{-/-} mouse model for investigating AD pathogenesis.

Atractylenolide I, a novel dual-target therapeutic candidate, is known for its strong neuroprotective and anti-atherosclerotic effects [47,48]. In the present study, AI administration not only reversed cognitive impairment but also orchestrated tissue repair via multiple mechanisms: inhibition of neuronal apoptosis, restoration of neuronal morphology, attenuation of amyloid plaque formation, and restoration of BBB integrity. These data indicated the potential of AI for the prevention and treatment of memory-related diseases [49]. Emerging evidence highlights the intricate interplay between lipid dysmetabolism and neurodegenerative pathologies. Abnormal cholesterol metabolism in arterial macrophages is the main cause of atherosclerosis, whereas this hardening of the arteries affects the blood supply to the brain and may contribute to the onset of AD. More importantly, we revealed that pathological lipid accumulation coincides with M1 macrophage polarization in the hippocampus—a phenomenon characterized by elevated expression of proinflammatory mediators (iNOS, IL-1β, TNF-α, and IL-6) and foam cell formation through excessive lipid phagocytosis [50]. Concurrently, AI attenuated cerebral lipid deposition through upregulation of LXR/ABCA1-mediated cholesterol efflux, thereby preventing macrophage-to-foam cell transformation [51].

To decipher the metabolic basis of these effects, untargeted metabolomics revealed AI-mediated normalization of dysregulated metabolites (such as betaine, malic acid, and urea), with arginine biosynthesis emerging as the most significantly modulated pathway. Other metabolites, such as N-methyl nicotinamide, trimethylamine oxide, and creatinine, have also been identified as being associated with the pathology of AD [52,53]. However, the cellular and molecular processes underlying these metabolites in AD remain to be fully explored. Previous research has demonstrated a connection between dysregulated arginine biosynthesis and the risk of developing both AD and atherosclerosis [54,55]. In light of this, it was unsurprising that the ApoE^{-/-} mice fed an HFD showed signs of arginine metabolism disruption in our study. On the basis of these findings, we further explored the underlying mechanisms and possible molecular targets of AI in the regulation of arginine biosynthesis.

ARG1 is the last hydrolysis enzyme in the urea cycle and hydrolyzes arginine to ornithine and urea. Although research suggesting that ARG1 directly regulates lipid metabolism is limited, compelling evidence shows that ARG1 competes with NOS for arginine, thereby limiting NO production [56], which increases fat accumulation [57,58]. Consistently, a potential link between NO deficiency and lipid accumulation in macrophages has been demonstrated. Moreover, as another key gene in the urea cycle within the arginine biosynthesis pathway, nNOS directly catalyzes the oxidation of arginine to NO and citrulline to drive the urea cycle. Strikingly, the urea concentrations halved in AI-treated mice—a reduction strongly correlated with ARG1 downregulation and consistent with AD-associated urea cycle disruption. Mechanistically, AI differentially regulated key enzymes, not only downregulating ARG1 to reduce urea production but also upregulating nNOS to increase neuroprotective NO synthesis. This targeted

modulation was further substantiated by molecular docking and SPR confirmation of direct AI-ARG1 binding ($K_D = 17.49 \mu\text{M}$), indicating that AI may modulate arginine biosynthesis and associated lipid metabolic disorders by selectively targeting ARG1, thereby alleviating the pathology of Alzheimer's disease in ApoE^{-/-} mice.

In summary, the present study demonstrates that AI alleviates AD pathologies in HFD-fed ApoE^{-/-} mice through resolution of lipid dyshomeostasis, suppression of neuroinflammation, and rebalancing of arginine-urea metabolism by regulating the ARG1/nNOS axis. Nonetheless, the cellular-level mechanisms by which ARG1 regulates arginine biosynthesis warrant further validation using macrophage-specific knockout models, and clinical translatability requires additional dose-response optimization.

Data Availability

The metabolomics data have been deposited in the Zenodo repository (<https://doi.org/10.5281/zenodo.19000654>). All other data can be accessed upon reasonable request from the corresponding author.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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