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Nut consumption as a therapeutic strategy to preserve brain function, attenuate neuropathology, and modulate cross-tissue microRNAs in a mouse model of Alzheimer's disease

Laura Panisello[1,2,3], Mireia Millet-Sigalat[4,5,6,7], Nil Novau-Ferré[1,2,3], Javier Mateu-Fabregat[1,2,3], Marina Carrasco[4,5,6,7], Miren Ettcheto[4,5,6,7], Elisa Félix-Soriano[1,2,3], Antoni Camins[4,5,6,7], Mònica Bulló[1,2,3,8*]

[1]Nutrition and Metabolic Health Research Group, Rovira i Virgili University, Reus, Spain.

[2]Institute of Health Pere Virgili (IISPV), Reus, Spain.

[3]Center of Environmental, Food and Toxicological Technology – TecnATox, Rovira i Virgili University, Reus, Spain.

[4]Department of Pharmacology and Therapeutic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain.

[5]Biomedical Research Network Center in Neurodegenerative Diseases (CIBERNED), Madrid, Spain.

[6]Institute of Neuroscience, Universitat de Barcelona, Barcelona, Spain.

[7]Department of Biochemistry and Physiology, Physiology Section, Faculty of Pharmacy and Food Science, Universitat de Barcelona, 08028 Barcelona, Spain.

[8]CIBER Physiology of Obesity and Nutrition (CIBEROBN), Carlos III Health Institute, 28029 Madrid, Spain.

***Corresponding author:**

Mònica Bulló. Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili (URV). C/Sant Llorenç 21, 43201 Reus, Spain. E-mail: monica.bullo@urv.cat

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Abstract

Nutritional modulation of brain metabolism is emerging as a key strategy for preventing Alzheimer's Disease (AD), with potential to influence key pathologies such as amyloid beta/β (Aβ) accumulation, tau phosphorylation, and neuroinflammation. However, the biological mechanisms linking diet, metabolism, and AD remain poorly understood. The aim of this study is to investigate the neuroprotective effects of a nut-enriched diet (NED) on AD-like pathology using APPswe/PS1dE9 (APP) transgenic mice, focusing on cognition, neuroinflammation, Aβ burden, and the potential regulatory role of circulating and brain-tissue specific microRNA (miRNA). APP and wild-type (WT) male mice were fed either a control diet (CD) or NED providing 10% of total energy from mixed nuts. Behavioral performance, Aβ deposition, glial activation, and synaptic integrity were assessed, alongside miRNA profiling in serum, cortex, and hippocampus. In APP mice, NED enhanced hippocampal-dependent memory, reduced microglia and astrocyte reactivity, decreased cortical and hippocampal Aβ plaque burden, and preserved dendritic spine density. Multi-compartment miRNA analyses revealed that NED modulated several AD-relevant miRNAs involved in insulin signaling, neuroinflammation, and synaptic function. These miRNA alterations correlated with improved cognitive outcomes and attenuated neuropathology, suggesting coordinated metabolic and molecular reprogramming in response to dietary intervention. A nut-enriched diet exerted significant neuroprotective effects in an AD mouse model, potentially mediated through coordinated miRNA regulation and related metabolic pathways. These findings support nut consumption as a feasible nutrition-based strategy for AD prevention and identify candidate miRNAs that may serve as biomarkers or mechanistic mediators at the intersection of diet, metabolism, and neurodegeneration.

Keywords: nuts, Alzheimer's disease, cognition, neurodegeneration, microRNAs.

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1. Introduction

Dementia is a major public health concern recognized by the World Health Organization (WHO), affecting over 55 million people and projected to rise to 139 million by 2050. Alzheimer's disease (AD), the most common form of dementia, accounts for 60-70% of cases and is one of the most devastating and costly diseases of this century (1–3).

AD is a neurological disorder characterised by an irreversible and progressive decline in cognitive functions, leading to loss of independence and a significant reduction in quality of life for patients and caregivers. The pathological hallmarks of AD include the neuronal accumulation of extracellular amyloid-β (Aβ) plaques and intracellular hyperphosphorylated tau (p-tau) tangles, which contribute to synapse loss (4,5). Additionally, activated microglia and reactive astrocytes drive a sustained neuroinflammatory response that exacerbates neuronal damage (6). However, the initial trigger for the Aβ and p-tau accumulation remains unclear. Consequently, there is growing interest in understanding the pathophysiology of AD, identifying key risk and protective factors, and discovering early biomarkers to support new treatments and prevention strategies (2,7,8).

Recent studies indicate lifestyle, particularly diet, as a significant contributor to AD onset (9). Nutritional interventions that support metabolic health and reduce neuroinflammation may offer a promising strategy to mitigate the risk of cognitive decline (10). Notably, higher intake of mono and polyunsaturated fatty acids has been consistently associated with slower rates of cognitive decline and reduced risk of developing AD (11). These compounds play an important role in regulating inflammation, oxidative stress and metabolic function, thereby influencing Aβ clearance, tau phosphorylation and the preservation of neuronal integrity (12). In this context, nuts, a natural source of unsaturated fatty acids, antioxidants and anti-inflammatory compounds, has emerged as a potentially effective dietary strategy to support brain health through the preservation of metabolic and neuronal homeostasis. However, evidence from

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intervention studies remains limited and inconclusive. A meta-analysis of randomised controlled clinical trials reported no significant effects of nut consumption on cognitive performance, highlighting important limitations in the existing literature, including short intervention durations, heterogeneous study designs and small sample sizes (13,14). Moreover, despite the proposed benefits of nuts, intervention studies specifically evaluating their preventive potential in the context of AD are scarce, and the molecular mechanisms mediating their effects remain largely unexplored.

Understanding how diet influences AD risk requires the identification of accessible and reliable early biomarkers to improve diagnostic and guide the development of targeted treatments. MicroRNAs (miRNAs), which regulate gene expression at the post-transcriptional level, have emerged as promising candidate, both as biomarkers and potential therapeutic targets for cognitive decline and neurological disorders (15–17). In the brain, miRNAs play critical roles in modulating neuronal differentiation, maturation, and synaptic plasticity, and have been implicated in key pathological processes of AD (18–20). Previous studies from our group have shown that a high-fat diet induces cognitive deficits in wild type (WT) mice, which were accompanied by significant alterations in miRNA expression profiles, suggesting a mechanistic link between dietary composition, epigenetic regulation, and neurodegenerative processes (21). Extending these findings, the present study aims to evaluate, for the first time, the impact of a nut-enriched diet on neurodegeneration in APP mice. Specifically, we investigate whether this dietary intervention modulates the expression of miRNAs in both circulating blood and brain tissue, thereby contributing to neuroprotection and offering potential molecular targets for early intervention in AD.

2. Experimental

2.1. Animals and Dietary Treatment

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A total of 63 male APPswe/PS1dE9 (APP) double transgenic mice and 60 WT littermates with the same genetic background (C57BL/6) were obtained from established breeding pairs at the institutional animal facilities (Faculty of Pharmacy and Food Sciences of the University of Barcelona). The number of mice used was based on previous studies, which showed changes in AD features across different treatments, to ensure enough animals to detect similar effects in this study. After weaning at 21 days old, APP and WT mice were randomly assigned to either a conventional chow diet (control diet, CD; ENVIGO, Madison, WI, USA) or a nut-enriched diet providing 10% of the energy from nuts, specifically containing walnuts, almonds and hazelnuts in a 2:1:1 proportion (nut-enriched diet, NED; ENVIGO, Madison, WI, USA). Diet compositions are detailed in Table S1. At 6 months, animals underwent glucose and insulin tolerance tests (GTT and ITT, respectively), followed by behavioural assessments. Animals were sacrificed, and blood and brain samples collected for analysis (Figure S1).

All animals were housed under controlled humidity and temperature conditions, with a 12-hours light-dark cycle and free access to food and water. Experimental procedures were conducted in accordance with the ethical guidelines established by the European Committee (approval number CEEA 626-25). The experimental protocol followed the "Principles of Laboratory Care" guidelines and was carried out following the European Communities Council Directive (2010/63/EEC).

2.2. Glucose and Insulin Tolerance Tests

Mice were fasted for 6 h and the tests were performed in a temperature-controlled room maintained at +28 °C. Blood glucose levels were measured via tail vein sampling using a standard glucometer (Accu-Chek® Aviva, Roche Diagnostics). For the GTT, mice received an intraperitoneal administration of glucose at a dose of 1g/kg body weight (Merck KGaA, Darmstadt, Germany). Blood glucose concentrations were measured at baseline and at 5, 15, 30 and 60 minutes post-injection. Three days later, the ITT was performed by administering

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insulin intraperitoneally at a dose of 0.75 IU/kg of body weight (Actrapid® Innolet®, Novo Nordisk A/S, Bagsvaerd, Denmark). Blood glucose levels were measured at baseline and at 15, 30, 45, 60 and 90 minutes after insulin administration (22).

2.3 Behavioural tests assessment

2.3.1. Morris Water Maze test (MWM)

Spatial learning and memory were assessed using the MWM as previously described (23), employing the SMART V3.0 video tracking system (Panlab Harvard Apparatus, Germany) and using between 19 and 24 animals per group, corresponding to a total of 85 animals across all tests. Briefly, a hidden white platform was submerged in a white-water tank (21 ± 2 °C) positioned in the centre of the northeast quadrant. The navigation phase was conducted over six consecutive days, during which mice underwent five training trials per day to locate the hidden platform using distal spatial cues. Each trial lasted a maximum of 60 seconds, during which the animal was allowed to search for the platform. If the mouse failed to find the platform within the allotted time, it was guided to the platform and left there for 30 seconds to reinforce spatial learning. The probe trial was conducted the day after the last training session to evaluate memory retention. On this day, the platform was removed, and each mouse was released from the southwest quadrant and allowed to swim for 60 seconds. This test evaluated the hippocampus-dependent spatial and learning memories.

2.3.2. Novel Object Recognition Test (NORT)

Recognition memory was evaluated by the NORT as previously described (24), in a controlled environment consisting of a circular open-field arena of 40 cm of diameter, surrounded by black curtains and maintained under constant illumination. The protocol included three phases: habituation, familiarization and testing. During habituation, mice individually explored the empty arena for 10 minutes across three consecutive days. On day four, during familiarization, two identical objects were placed in the centre of the arena, and each mouse explored for 10

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minutes. After 24 h, mice returned to the area where one of the previous objects remained unchanged, and the other was replaced with a novel object. Mice were allowed to explore the arena for 10 minutes, during which behaviour was recorded and a discrimination index (DI) calculated as the difference in time exploring the novel versus the familiar object, relative to total exploration time. The test was conducted using between 23 and 26 animals per group.

2.4. Neuroinflammation evaluation

Neuroinflammation in the brain was assessed by evaluating microglial and astrocyte reactivity using immunofluorescence staining for ionized calcium-binding adaptor molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP) in 5-7 animals per group. IBA1 is selectively expressed by microglia and macrophages and is widely used as a microglial marker (25,26). GFAP concentration is commonly used as a proxy for astrocyte reactivity (27). Coronal brain sections (20 µm thick) were obtained using a cryostat (Leica Microsystems, Wetzlar, Germany) and stored in a cryoprotectant solution at -20ºC until use. Free-floating sections were rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.35), followed by PBS-T (PBS 0.1M with 2% of Triton X-100). Sections were then incubated in blocking solution (10% fetal bovine serum (FBS), 1% Triton X-100, PBS 0.1M + 0.2% gelatine) for 1-2 hours at room temperature. After blocking, sections were washed with PBS-T and incubated overnight at 4ºC with primary antibodies against IBA1 and GFAP (Table S2). The next day, sections were washed again with PBS-T and incubated for 2 hours at room temperature with the secondary antibody (Table S2). Cell nuclei were stained with Hoechst (0.1 µg/mL, Sigma-Aldrich, St Louis, MO, United States) for 8 minutes in the dark at room temperature, followed by a final PBS wash. Samples were mounted on Superfrost microscope slides using Fluoromount medium and left to dry overnight. Images were acquired using an epifluorescence microscope (BX61 Laboratory Microscope, Olympus, Melville, NY Olympus America Inc.) and quantified with ImageJ.

2.5. Brain β-amyloid accumulation

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Amyloid β1-42 ($A\beta_{1-42}$) levels in the cerebral cortex from 8-10 animals per group were measured using enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instruction (ThermoFisher Scientific; KHB3441, USA). Data are expressed in pg/µg of protein. To evaluate not only the concentration of Aβ but its tendency to form plaques, coronal brain sections from 5-7 animals per group were stained with Thioflavin-S diluted with PBS 0.1 mol/L on a 0.0033% concentration and incubated for 8 minutes in the dark at room temperature. Samples were mounted on Superfrost. Aβ plaques were visualized and counted under an epifluorescence microscope (BX61, Olympus) and quantified using ImageJ. Data are expressed as number of Aβ plaques/slide.

2.6. Hippocampal dendritic spine density analysis

Five to six mice per group were sacrificed by cervical dislocation, and their brains were processed according to the instructions from FD GolgiStainTM Kit (FD Neurotechnologies, Inc.). Dendritic spine quantification was conducted in 5 neurons from two hippocampal regions: the dentate gyrus (DG) and the CA1. In the DG, secondary branches from the terminal fragment and dendritic ramifications were analysed, while in CA1, two neuronal compartments were evaluated: CA1 basal and CA1 apical dendrites.

Images were acquired using Leica Thunder Microscope (Leica Microsystems, Germany). All quantifications were performed over 30 µm dendritic segments, excluding the first 20 µm of each dendrite. Spine density is expressed as the number of spines per 30 µm of dendrite.

2.7. MicroRNA expression

2.7.1. RNA extraction

For serum RNA, 400 µL of sample were processed using the Plasma/Serum RNA Purification Midi Kit (Norgen Biotek, Canada) for sncRNAseq screening, and the miRVANA PARIS kit (Thermo Fisher Scientific, USA) for RT-qPCR analysis. Tissue RNA was extracted from approximately 30 mg of cortex and hippocampus samples using the PureLink RNA Extraction

Mini Kit (Thermo Fisher Scientific, USA). Prior to extraction, the tissue was thoroughly homogenized using an IKA ULTRA TURRAXTM T18 Digital Disperser (IKA Werke GmbH & Co, Staufen, Germany). The exogenous positive control cel-miR-39-3p from *Caenorhabditis elegans* was added to each sample prior to RNA extraction. The quantity and quality of isolated RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with 2 µL of each sample.

2.7.2. Small non-coding RNA high-throughput sequencing

Samples from serum, cortex and hippocampus were randomly selected to perform small non-coding RNA (sncRNA) high-throughput sequencing. 5 µL of RNA isolations, containing 400-1000 ng of total RNA, were used to prepare sequencing libraries using the Truseq small RNA Library Preparation Kit (Illumina, San Diego, USA). Then, Agilent DNA High Sensitivity ScreenTape Kit (Agilent Technologies, Santa Clara, USA) was used to assess the DNA fragment size and concentration. Samples of 450 pM of DNA were used to perform the massive sequencing using the Nextseq2000 equipment (Illumina, San Diego, USA). To select microRNAs for validation, sncRNA-Seq data were analysed for differential abundance (DA) between genotypes using three different methods: DESeq2, edgeR normalized by Trimmed Mean of M-values method and edgeR normalized to cel-miR-39-3p expression (28). The microRNA selection criteria were based on the presence of significant differences in the DA (at least with the DESeq2 method) and having previously reported associations with AD features. A total of 22 miRNAs were selected for validation through RT-qPCR across all samples (mmu-let-7b-3p, mmu-miR-122-5p, mmu-miR-153-3p, mmu-miR-187-3p, mmu-miR-187-5p, mmu-miR-214-3p, mmu-miR-218-2-3p, mmu-miR-3057-5p, mmu-miR-338-5p, mmu-miR-3547-3p, mmu-miR-429-3p, mmu-miR-451a, mmu-miR-6240, mmu-miR-6516-5p, mmu-miR-669a-5p, mmu-miR-669o-5p, mmu-miR-702-3p, mmu-miR-19a-3p, mmu-miR-98-5p, mmu-miR-29c-3p, mmu-miR-338-5p, mmu-miR-34a-5p), along with the

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exogenous positive control cel-miR-39-3p and the negative control ath-miR-159a from *Arabidopsis thaliana*.

2.7.2. miRNA rt-PCR

Total RNA was reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Darmstadt, Germany). A total of 22 miRNAs, together with positive and negative controls, were quantified by real-time PCR using specific primers for TaqMan Gene Expression Assays (Thermo Fisher Scientific, USA). All reactions were performed in duplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and data were analyzed using Design & Analysis Software v2.6.0 (Applied Biosystems, Thermo Fisher Scientific).

miRNAs with Ct > 35 were considered not expressed. miRNAs with more than 65% missing values were excluded for subsequent analyses. Following these criteria, miRNAs 3057-5p, 6516-5p and 187-5p were excluded from all analyses; furthermore, miRNAs 153-3p, 214-3p and 3547-3p were excluded only from the serum analysis; while miRNAs 429-3p and 669a-5p were excluded from both cortex and hippocampus analyses. Samples with more than 65% unexpressed miRNAs or presenting ath-miR-159 (negative control) expression were also excluded. Ct values were first normalized to cel-miR-39-3p. A second normalization was performed by mean-centering the values using their respective means in control mice. Differential expressions were calculated as log2 fold change. Finally, values greater than three times the interquartile range (IQR) above the third quartile or below the first quartile were considered extreme outliers and removed from the analysis.

2.8. Statistical analysis

All results are presented as mean ± SE. The normality of the data distribution was assessed with the Shapiro-Wilk test. Differences between groups were evaluated through the Student's

t test or Mann-Whitney test according to, respectively, normal and non-normally distributed data. Correlation analyses between miRNA expression and results from the GTT, ITT, cognitive performance from MWM and NORT, and cortical Aβ1–42 levels, were conducted using Pearson's or Spearman's correlation coefficients depending on data distribution. Correlation analyses were performed specifically within the control groups of each genotype, to better characterize baseline associations independent of dietary intervention; and results are presented as correlation coefficients (r) and p-value (p). All analysis were carried out using R version 4.2.3 (R Foundational for Statistical Computing in Vienna, Austria).

Finally, the functional enrichment analysis was performed to identify the target genes and significant pathways leaded by the differentially expressed miRNAs. The target genes from each miRNA were found on TargetScan platform (29), network diagram of different miRNAs and their target genes was generated using miRNet platform (30) and significant KEGG pathways and Gene Ontology (GO) functions for the target genes were found in the WEB-based GEne SeT AnaLysis Tool (WebGestalt) (31).

3. Results

3.1. APP genotype exhibits reduced glucose and insulin tolerance

Although a significant difference in food intake (Figure 1A) was observed between the APP/CD and APP/NED groups ($p < 0.05$), no significant differences were found in body weight (Figure 1B). Regarding glucose and insulin metabolism (Figure 1C-F), mice carrying the APP genotype showed significantly impaired glucose tolerance compared to WT ($p < 0.05$). A similar trend was observed in the insulin tolerance test, though it did not reach statistical significance ($p = 0.09$).

3.2. Nuts intake improved hippocampal memory

As expected, mice with the APP genotype showed significantly poorer performance in both the MWM and NORT compared to WT controls ($p < 0.005$). Furthermore, within the APP

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genotype, mice fed with the NED demonstrated enhanced spatial and learning abilities, requiring significant less time to locate the platform during the MWM test ($p < 0.005$) (Figure 2A, B). Moreover, on the last day of MWM testing (day 7), the APP/NED mice made more entries to the platform ($p < 0.005$) (Figure 2C) and spent more time in the platform zone ($p < 0.005$) (Figure 2D). Similarly, the NORT revealed significantly increased ability to discriminate the novel object (DI) in APP/NED mice compared to APP/CD controls ($p < 0.005$) Figure 2E).

3.3. Nuts intake attenuated microglia and astrocyte reactivity

Microglia and astrocyte neuroinflammatory responses were evaluated through immunofluorescence staining for IBA1 (Figure 3A-E) and GFAP (Figure 3F-J) respectively. APP/CD showed significantly increased microglial and astrocytic reactivity compared to WT/CD controls ($p < 0.005$ and $p < 0.05$, respectively). On the contrary, APP mice fed with the NED showed lower levels of both inflammatory markers ($p < 0.005$ for microglia, $p = 0.05$ for astrocytes) compared to APP/CD. A similar reduction in microglial activation was also observed in the WT/NED mice in comparison to the WT/CD ($p < 0.05$).

3.4. Nuts intake reduced β-amyloid plaques in hippocampus and cortex

Cortical $A\beta_{1\text{-}42}$ concentrations were significantly higher in APP/CD mice compared to the WT/CD controls ($p < 0.005$), however the nuts intervention had no significant effect on $A\beta_{1-42}$ levels (Figure 4C). In contrast, when assessing Aβ plaque burden using Thioflavin-S staining rather than protein concentration, APP/NED mice showed a reduction in plaque number in the cortex (Figure 5B, $p < 0.005$) and a trend towards reduction in the hippocampus (Figure 4A, $p = 0.07$) in comparison to APP/CD.

3.5. Nuts intake improved neuronal preservation

Across all hippocampal regions, APP/CD mice showed a significantly greater loss of dendritic spines in comparison to WT/CD controls ($p < 0.005$ for all the examined hippocampal regions).

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Conversely, within the APP genotype, mice fed with the NED showed significant grater preservation of dendritic spines density across all hippocampal regions ($p < 0.05$ for DG ramifications and terminal fragment (Figure 5A-J), $p < 0.05$ for CA1 basal fragments (Figure 5K-O), $p < 0.005$ for the CA1 apical fragment (Figure 5P-T)).

3.6. Nuts intake modulates miRNAs differentially expressed in the APP genotype

In serum, miR-122-5p, miR-6240, miR-669a-5p and miR-98-5p were significantly upregulated in APP/CD mice compared to the WT/CD mice, while miR-338-3p was notably downregulated (Figure 6A). Interestingly, within the APP genotype, miR-669a-5p and -98-5p levels were significantly reduced in animals fed with the NED, while miR-338-3p showed a trend toward increased expression. Furthermore, in the APP genotype, the NED significantly downregulated the expression of miR-187-3p and miR-429-3p, whereas miR-451a was significantly upregulated (Figure 6B). In the WT genotype, the NED intervention led to a significant increase in serum levels of miR-29c-3p and miR-98-5p (Figure 6C). Interestingly, correlation analyses revealed that higher serum expression of miR-122-5p ($r = 0.57$, $p < 0.005$), miR-669a-5p ($r = 0.45$, $p < 0.05$), and miR-98-5p ($r = 0.54$, $p < 0.005$) correlated with altered cognitive decline observed in MWM. In contrast, elevated miR-338-3p expression correlated with reduced cortical Aβ accumulation ($r = -0.69$, $p < 0.005$) and improved MWM performance ($r = -0.46$, $p < 0.05$) (Figure 7A).

Regarding miRNA expression in the cortex, 9 miRNAs (7b-3p, 122-5p, 153-3p, 29c-3p, 338-5p, 3547-3p, 451a, 6240 and 702-3p) were significantly upregulated in the APP/CD group in comparison to the WT/CD (Figure 6E). Among these, miR-122-5p and miR-451a levels were significantly reduced in APP mice fed with the NED in comparison to the APP/CD group (Figure 6F). Additionally, in the APP genotype, the NED also downregulated the expression of miR-218-2-3p (Figure 6F). In WT mice, the NED intervention significantly increased the cortical expression 4 miRNAs (19a-3p, 214-3p, 29c-3p and 451a) (Figure 6G). Correlation

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analyses further revealed that higher cortical expression of miR-let-7b-3p in control groups correlated with greater cortical Aβ accumulation ($r = 0.60$, $p < 0.05$) and increased latency in the MWM ($r = 0.45$, $p < 0.05$) (Figure 7B). Similarly, elevated levels of miR-122-5p ($r = 0.43$, $p < 0.05$) and miR-451a ($r = 0.54$, $p < 0.005$) correlated with worse MWM performance, while increased expression of miR-153-3p correlated with grater Aβ burden ($r = 0.66$, $p < 0.05$). In addition, miR-338-5p ($r = 0.59$, $p < 0.005$), miR-3547-3p ($r = 0.45$, $p < 0.05$), miR-6240 ($r = 0.60$, $p < 0.005$) and miR-702-3p ($r = 0.79$, $p < 0.005$) in cortex correlated with poorer glucose tolerance (Figure 7B).

In the hippocampus, miR-19a-3p and miR-451a were significantly downregulated in the APP/CD group in comparison to the WT/CD (Figure 6I). Interestingly, within the APP genotype, both miRNAs were further reduced in response to the NED (Figure 6J). The expression of miR-19a-3p and miR-451a in hippocampus correlated with less Aβ accumulation ($r = -0.83$, $p < 0.005$; $r = -0.66$, $p < 0.05$ respectively) and enhanced MWM performance ($r = -0.66$, $p < 0.005$; $r = -0.47$, $p < 0.05$ respectively), and the miR-19a-3p further correlated with enhanced insulin tolerance ($r = -0.46$, $p < 0.05$) and cognitive performance measured by NORT ($r = 0.43$, $p < 0.05$) (Figure 7C). Furthermore, miR-338-5p was significantly upregulated in the APP/CD group in comparison to the WT/CD (Figure 6I). In the APP genotype, the NED upregulated the expression of let-7b-3p, miR-6240 and miR-702-3p (Figure 6J). From them, let-7b-3p and miR-702-3 expression correlated with enhanced results in MWM ($r = -0.49$, $p < 0.05$; $r = -0.47$, $p < 0.05$ respectively), and let-7b-3p was further negatively correlated with Aβ accumulation ($r = -0.57$, $p < 0.05$) (Figure 7C). Moreover, in the WT genotype, the NED significantly downregulated the expression of 4 miRNAs (153-3p, 338-3p, 34a-5p and 451a) (Figure 6K).

Overall, miR-338-3p, miR-669a-5p, miR-98-5p, miR-122-5p, miR-19a-3p and miR-mi451a exhibited significant differential expressions between genotypes under the CD condition.

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Notably, in the APP genotype, the NED significantly modulated the expression of these miRNAs, shifting their expression profile toward those observed in the WT genotype. A minimal gene-miRNA interaction analysis using miRNet platform identified direct interactions between the 6 differentially expressed miRNAs and 7 hub genes (Figure 8A). Target prediction using the TargetScan database yielded a total of 4,535 potential target genes. KEGG pathway enrichment analysis for these genes, preformed via WebGestalt, revealed significant overrepresentation of pathways related to type 2 diabetes mellitus (FDR <0.05), axon guidance (FDR <0.001), insulin signalling (FDR <0.01), and AMPK signalling (FDR <0.05) (Figure 8B). Furthermore, GO enrichment analysis indicated that the most significantly enriched biological processes, molecular functions and cellular components were related to synaptic development, neuronal function, and plasticity (Table S4).

4. Discussion

The results of this study demonstrate, for the first time, that a targeted nutritional intervention with mixed nuts effectively protects against the development of key pathological and functional hallmarks of AD in APP mice. Specifically, mice receiving the nuts intervention from an early age exhibited significant improvements in cognitive performance, along with attenuated glial activation and astrocyte reactivity, as evidenced by reduced IBA1 and GFAP concentrations, reduced Aβ plaque accumulation, and preservation of synaptic integrity, indicated by increased dendritic spine density. The dietary intervention provided nuts at 10% of total energy intake which, when considering the metabolic rate, corresponds to the human equivalent of the recommended daily intake of approximately 30 g/day (32). Together, these findings support regular nut intake as a promising dietary strategy to delay or prevent cognitive decline and AD-related pathology.

Consistent with our findings, epidemiological studies in large cohorts show that regular nut consumption is associated with healthy aging, preserved cognitive and physical function, and

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improved mental health outcomes (33). In particular, a daily intake of 3 or more handfuls of nuts has been associated with a 12% lower risk of all-cause dementia over 7.1 years of follow-up, supporting a potential protective role of nuts in the prevention of neurodegenerative diseases (34). Evidence from randomized controlled trials has been less consistent. The largest trial to date examining the cognitive effects of a 2-year walnut intervention in cognitively healthy older adults did not detect significant improvements on global cognition, although complementary brain MRI and post-hoc site-specific results suggested that walnut consumption may delay cognitive decline in individuals at higher baseline risk (35). Similarly, a recent meta-analysis found no overall cognitive benefit of nut consumption, while highlighting substantial heterogeneity in study design, population characteristics, and intervention doses (13). These discrepancies suggest that null findings at the population level may mask biologically meaningful effects that are evident under controlled experimental conditions. Indeed, pre-clinical studies consistently demonstrate that lipidic bioactive compounds commonly found in nuts reduce Aβ and p-tau burden while preserving dendritic spine structure and synaptic function (36–38). Similarly, studies in mouse models show beneficial effects of walnuts, walnut oil and walnut-derived peptides on working memory, learning and cognitive function (39–41). These preclinical findings provide biological plausibility for the protective associations observed in epidemiological studies, suggesting that the neuroprotective role of nut consumption may be explained by their unique nutritional composition matrix, rich in unsaturated fatty acids, polyphenols, vitamins (e.g., vitamin E and folate), minerals (e.g., magnesium and zinc), and fiber. Similarly, bioactive compounds present in nuts, such as polyphenols and omega-3 polyunsaturated fatty acids, have shown promising effects in animal models and clinical studies, including reductions in Aβ levels, improvements in cognition, and modulation of signaling pathways implicated in AD (42). These effects are similar to our findings on reduced neuroinflammation and agree with AD studies showing that

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polyunsaturated fatty acids and antioxidants help reduce microglial activation, oxidative stress, and support synapses.

Rather than acting through isolated nutrients, mounting evidence indicates that synergistic interactions among these compounds confer greater health benefits than single-component supplementation (43). This is consistent with an umbrella review reporting associations between nut intake and reduced risk of several chronic diseases and mortality outcomes, alongside beneficial modulation of intermediate biomarkers related to inflammation and insulin metabolism (44). These systemic effects are increasingly recognized as contributors to brain health and modulators of AD progression, supporting the rationale that a dietary intervention targeting multiple physiological pathways may be more effective than reductionist approaches (44). Among these mechanisms, attenuation of neuroinflammation appears particularly relevant. Chronic activation of microglia and astrocytes accelerates disease progression by exacerbating Aβ and tau pathology. In the present study, APP mice receiving the nut-enriched diet displayed significantly reduced glial activation compared to controls, suggesting a dampening of neuroinflammatory responses that is strongly implicated in AD pathology. These findings are consistent with previous studies showing that omega-3 fatty acids, one of the main lipid components of nuts, inhibit microglial activation and reduce inflammatory markers (45). Additionally, nuts and their nutritional components are well established as modulators of glucose and insulin metabolism (46,47). Given that impaired glucose homeostasis and insulin resistance have been linked to AD, these metabolic benefits may also contribute to reduced disease risk. However, no significant differences were observed in glucose or insulin tolerance tests between dietary intervention groups. This is consistent with a clinical trial conducted in older adults with overweight or obesity, which reported no changes in peripheral insulin sensitivity following nut consumption but demonstrated improvements in brain insulin

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sensitivity (48). These findings suggest that the absence of peripheral metabolic effects does not preclude beneficial effects on brain metabolism (49).

The miRNA profiling identified several miRNAs as potential mediators of the neuroprotective effects of nut consumption. Thirteen miRNAs were differentially expressed between the APP and WT genotypes in plasma or brain regions, highlighting their potential as mechanistic contributors and minimally invasive biomarkers for AD. Increased miR-122-5p and miR-6240 were consistently upregulated in both plasma and cortex of APP mice, suggesting a systemic disease-related dysregulation. In contrast, miR-338-3p was downregulated in plasma but upregulated in both the cortex and hippocampus, suggesting a region-specific regulation. Lower serum levels of miR-338-3p correlated with increased latency in the MWM memory test and grater cortical Aβ accumulation. Importantly, 6 out of the 13 miRNAs were significantly modulated in APP mice following the nut-enriched diet, indicating a partial restoration of disease-altered miRNA expression. Plasma levels of miR-669a-5p and miR-98a-5p, elevated in APP mice compared to WT controls, were significantly downregulated after the NED, suggesting their involvement in nut-mediated neuroprotection. Higher levels of both miRNAs correlated with increased latency in MWM, consistent with previous *in vitro* studies demonstrating that downregulation of miR-98-5p can alleviate Aβ accumulation and neuronal apoptosis (50).

Cortical levels of miR-122-5p and miR-451a, both elevated in APP mice, were significantly downregulated following the NED intervention. Notably, higher miR-122-5p expression in serum and cortex correlated with poorer memory performance, as assessed by the MWM and NORT. These findings are consistent with clinical evidence showing elevated serum levels of miR-122-5p in AD patients and its role in neurodegeneration and metabolic disruption of insulin sensitivity (51,52). miR-451a showed a complex pattern: cortical expression correlated with increased latency in MWM, while hippocampal expression correlated with reduced

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latency in MWM and lower cortical Aβ burden. This dual role agrees with literature, some studies report reduced miR-451a in CSF and brain cells from APP mice (53), while others find it elevated in the serum of AD patients (54). In contrast, hippocampal levels of miR-19a-3p, reduced in APP mice compared to WT, were significantly upregulated after nut intake. Reduced hippocampal levels of miR-19a-3p correlated with impaired insulin tolerance, increased latency and decreased DI in memory assessments (MWM and NORT, respectively) and increased accumulation of cortical Aβ. These findings are supported by clinical evidence showing an inverse correlation between miR-19a-3p levels in cerebrospinal fluid and Aβ plaque density in patients with AD (55). Additionally, reduced miR-19a-3p expression has been reported in diabetic patients, with plasma levels negatively correlated with the blood glucose concentrations, suggesting a role in systemic glucose regulation (56). However, the role of miR-19a-3p in neurodegeneration remains controversial, as other studies have reported its upregulation in APP mice (21).

These results suggest that miRNAs act as context- and region-specific modulators of AD pathology, shaped by metabolic and inflammatory states. The capacity of the NED to regulate their expression suggests that nutritional interventions may alleviate AD pathology and normalize disease-associated miRNA profiles. Notably, diet can influence miRNA expression through multiple mechanisms, including changes in transcription, miRNA stability, posttranscriptional modifications, and interaction with RNA binding proteins and other non-coding RNAs (57). These diet-regulated miRNA profiles can post-transcriptionally modulate key gene networks involved in neurodegeneration (58). In the context of AD, miRNAs are known to influence critical pathological processes, including neuroinflammation, Aβ clearance and tau phosphorylation (59,60). In line with this, our *in silico* analysis indicated that the modulated miRNAs including miR-338-3p, miR-669a-5p, miR-98-5p, miR-122-5p, 19a-3p and miR-mi451a target pathways related to insulin resistance, synaptic function and oxidative

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stress, processes that are central to AD pathophysiology. Altogether, the dynamic and region-dependent expression of miRNAs underscores their potential as mechanistic mediators and minimally invasive biomarkers for early detection and dietary prevention strategies in AD.

A major strength of this study lies in the comprehensive evaluation of neurodegeneration, integrating structural, functional and molecular assessments. Structural alterations were quantified through Aβ accumulation, neuronal preservation, and neuroinflammation, while brain function was evaluated using both the MWM and the NORT, which are validated learning and memory tests. Another notable strength is the integration of miRNA profiling across serum, cortex, and hippocampus, which offers an integrative perspective of systemic and central dietary modulation of AD-related miRNAs. Moreover, the selection of a miRNA panel based on sncRNA-Seq enabled the validation of novel miRNAs candidates potentially linking diet to neurodegenerative processes. Despite these strengths, several limitations should be acknowledged. Although the GTT and ITT provide insight into peripheral metabolism, they do not capture brain insulin sensitivity, which is more relevant to neurodegeneration. The translational value of the findings is also limited due to the use of a controlled genetic model and the exclusive use of male mice; future studies should include females to explore sex-specific effects. Regarding miRNA analyses, the identified candidates are promising but require further validation of their gene targets and pathways to confirm functional relevance and clarify the molecular mechanisms underlying the observed neuroprotective effects. Finally, because this study relies on endpoint measurements, it is not possible to determine whether the observed changes in miRNA expression are a cause or a consequence of the improvements in AD-related outcomes.

5. Conclusion

This study demonstrates that regular nut consumption can delay or prevent Alzheimer's disease-related pathology and cognitive decline in APP mice. The nut-enriched diet preserved

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cognitive function performance, reduced Aβ accumulation, decreased neuroinflammation and maintained synaptic integrity. Importantly, the intervention also modulated the expression of several miRNAs across serum, cortex and hippocampus, partially restoring disease-altered miRNA profiles and correlating with key pathological and behavioural outcomes. These findings suggest that nuts may exert neuroprotective effects through both structural preservation and systemic molecular regulation, particularly via miRNAs implicated in neuroinflammation, metabolism, and synaptic function. Overall, this study supports the role of nuts as a feasible, non-pharmacological approach to prevent or slow neurodegeneration, and highlights the potential of miRNAs as both mechanistic mediators and minimally invasive biomarkers in the context of dietary interventions targeting Alzheimer's disease.

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L Panisello: Investigation, Data curation, Formal analysis, Visualization, Writing - original draft. M Millet: Methodology, Investigation, Writing - review & editing. N Novau-Ferré, J Mateu-Fabregat: Investigation, Data curation, Formal analysis, Visualization, Writing - original draft. M Carrasco, M Ettcheto: Methodology, Investigation, Writing - review & editing. E Félix-Soriano: Writing - review & editing. A Camins: Resources, Supervision, Writing - review & editing. M Bulló: Conceptualization, Methodology, Investigation, Data curation, Resources, Supervision, Funding acquisition, Visualization, Writing - Original Draft, Writing - review & editing. All authors approved the final manuscript and agreed to be accountable for all aspects of the work.

Conflicts of Interest

The authors declare no conflicts of interest.

Availability of data and materials

Data will be available upon request.

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Figures caption

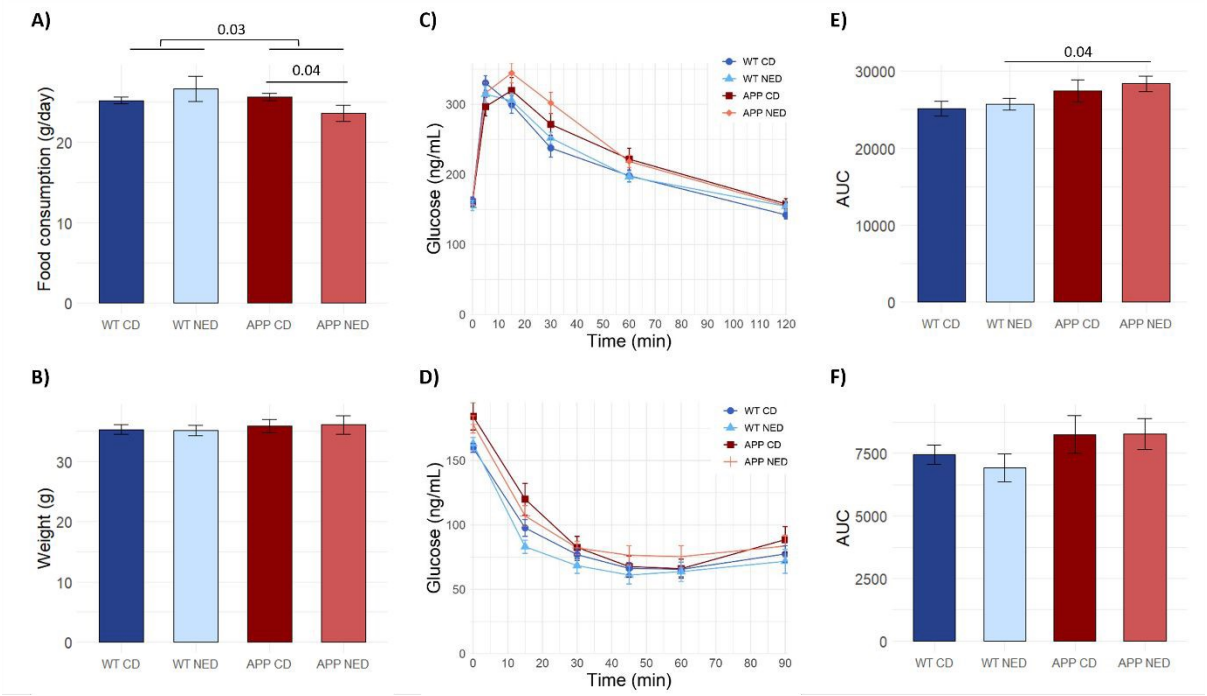

Figure 1. Differences in food consumption, weight, glucose and insulin metabolism in mice according to the genotype and intervention group. (A) Differences in food consumption (g/day) among intervention groups (n = 16 WT/CD, 4 WT/NED, 17 APP/CD and 7 APP/NED). (B) Differences in body weight (g) between intervention groups (n = 25 WT/CD, 29 WT/NED, 25 APP/CD and 25 APP/NED). Blood glucose levels along the Glucose (C, n = 22 WT/CD, 23 WT/NED, 22 APP/CD and 19 APP/NED) and Insulin (D, n = 23 WT/CD, 14 WT/NED, 21 APP/CD and 21 APP/NED) Tolerance Tests. Area under the curve (AUC) in the Glucose (E) and Insulin (F) Tolerance Tests according to experimental groups. Results are represented as mean ± SE, and differences between experimental groups were assessed by Student's T test or Mann-Whitney test according to normality. Abbreviations: WT, wild-type; APP, APP/PS1 transgenic mice mutation; CD, control diet; NED, nut-enriched diet; AUC, area under the curve.

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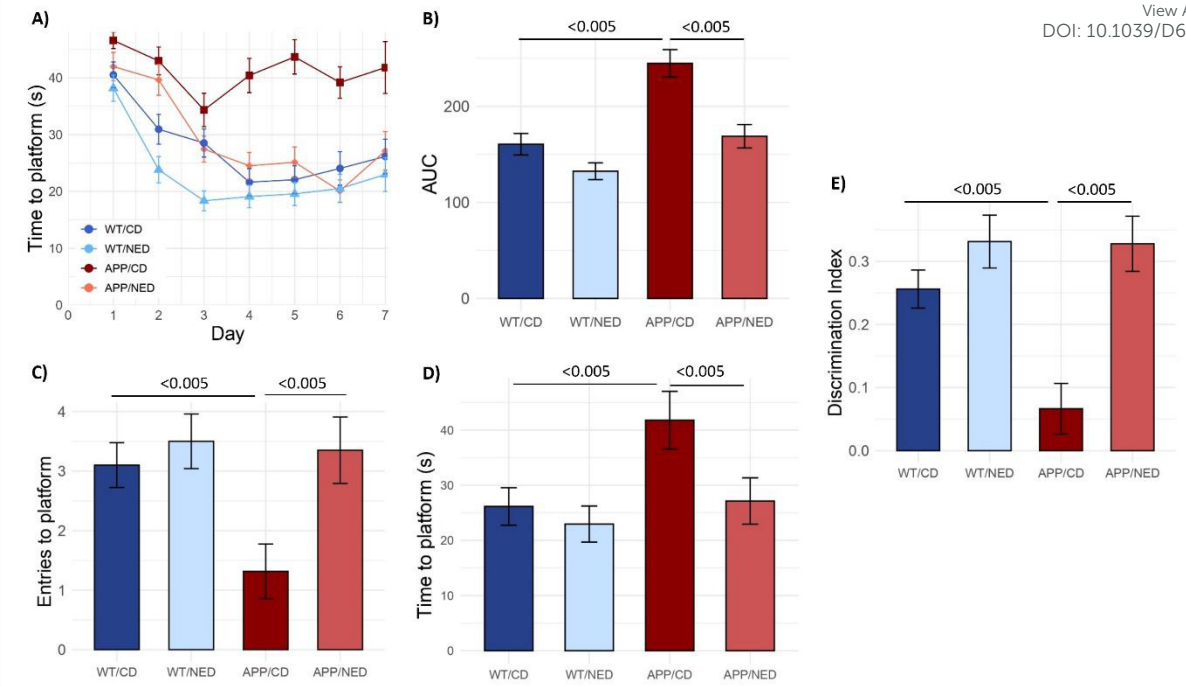

Figure 2. Differences in hippocampal memory tests performance. (A) Learning curve on the MWM test (n = 20 WT/CD, 24 WT/NED, 19 APP/CD and 20 APP/NED), represented as time spent reaching the platform in different testing days. (B) Area under the curve (AUC) from the MWM test performance across testing days. (C) Entries to platform on the last MWM testing day (day 7). (D) Time spent on the platform zone on the last MWM testing day (day 7). (E) NORT performance, measured as discrimination index (n = 23 WT/CD, 26 WT/NED, 23 APP/CD and 24 APP/NED). Results are represented as mean ± SE, and differences between experimental groups were assessed by Student's T test or Mann-Whitney test according to normality. Abbreviations: WT, wild-type; APP, APP/PS1 transgenic mice mutation CD, control diet; NED, nut-enriched diet; AUC, area under the curve; MWM, Morris Water Maze test; NORT, Novel object recognition test.

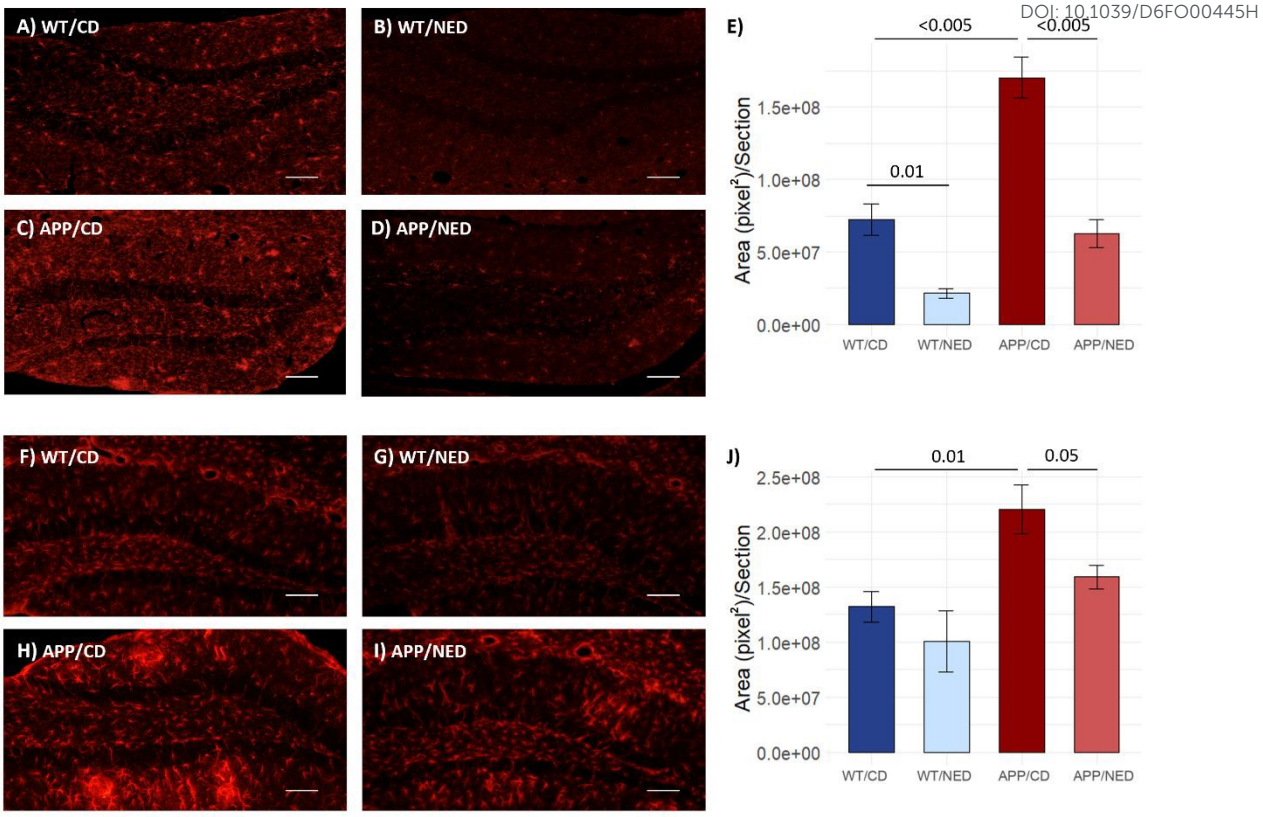

Figure 3. Inflammatory responses of microglia and astrocytes. Detection images of microglia (A-D) and astrocytes (F-I), scale bar: 50 µm. Fluorescence intensity quantification with IBA1 (E) and GFAP (J). n = 5 WT/CD, 5 WT/NED, 5APP/CD and 7 APP/NED. Results are presented as mean ± SE, and differences between experimental groups were assessed by Student's T test or Mann-Whitney test according to normality. Abbreviations: WT, wild-type; APP, APP/PS1 transgenic mice mutation; CD, control diet; NED, nut-enriched diet.

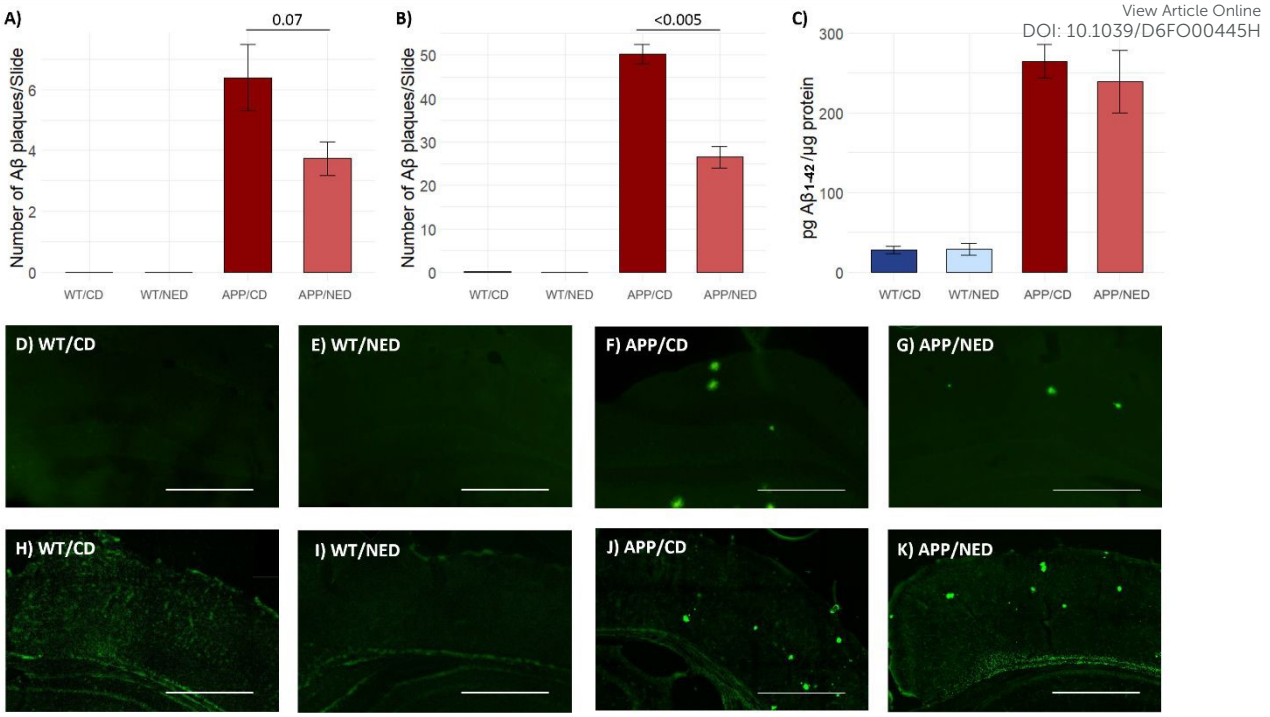

Figure 4. Number of amyloid β (Aβ) plaques in hippocampus (A) and cortex (B) tissues, assessed using thioflavin staining, and amyloid β1-42 levels (pg of Aβ/µg of protein) in cerebral cortex (C), assessed by ELISA method. Illustrative images of Aβ plaques in the hippocampus (D-G) and cortex (H-K), scale bar: 100 µm. N = 5 WT/CD, 5 WT/NED, 5 APP/CD, 7 APP/NED. Results are presented as mean ± SE, and differences between experimental groups were assessed by Student's T test or Mann-Whitney test according to normality. Abbreviations: WT, wild-type; APP, APP/PS1 transgenic mice mutation; CD, control diet; NED, nut-enriched diet; Aβ, amyloid β; ELISA, enzyme-linked immunosorbent assay.

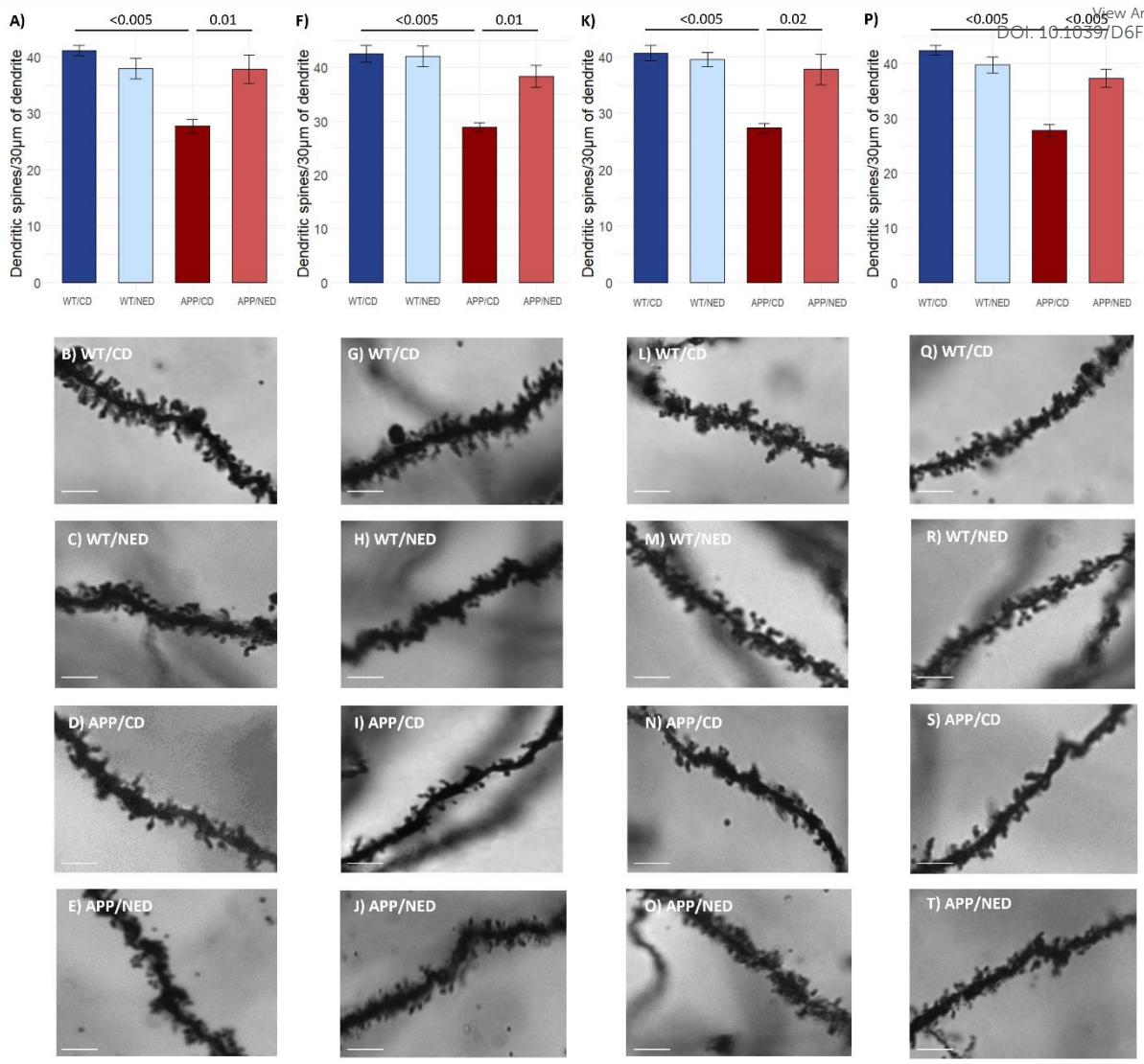

Figure 5. Quantification of dendritic spines for 30 µm of dendrite in different hippocampal regions (n = 6 WT/CD, 5 WT/NED, 6 APP/CD, 5 APP/NED): the terminal fragment of Dentate Gyrus (A-E), the ramification of the Dentate Gyrus (F-J), the basal fragment of CA1 region (K-O), and the apical fragment of the CA1 region (P-T). Scale bar = 5 µm. Results are presented as mean ± SE, and differences between experimental groups were assessed by Student's T test or Mann-Whitney test according to normality. Abbreviations: WT, wild-type; APP, APP/PS1 transgenic mice mutation; CD, control diet; NED, nut-enriched diet.

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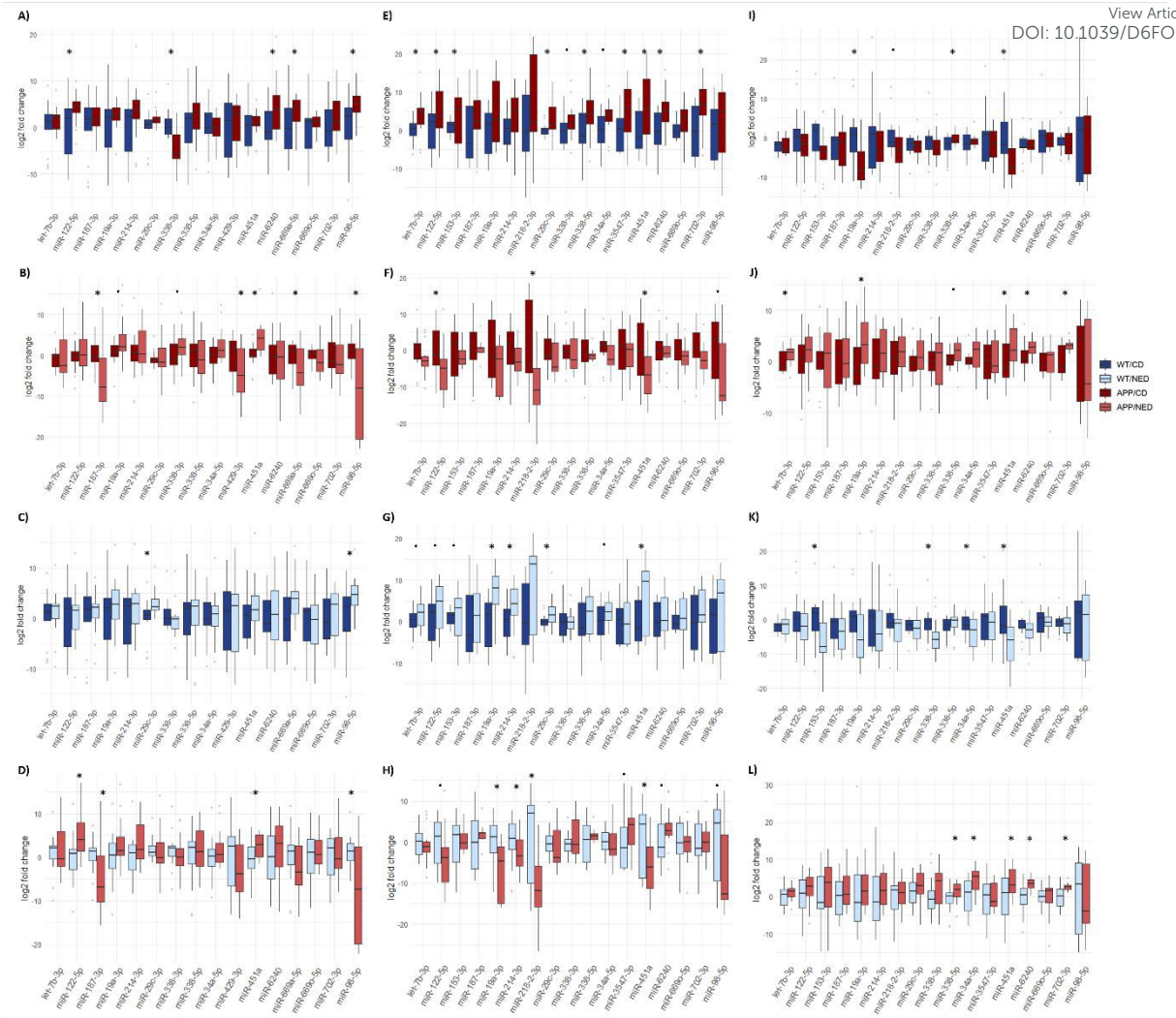

Figure 6. Differences in the expression of miRNAs in serum (A-D), cortex (E-H) and hippocampus (I-L) tissues across experimental groups (n = 17-24/group). Differences between experimental groups were assessed by Student's T test or Mann-Whitney test according to normality (· p-value < 0.1, * p-value <0.05). Abbreviations: WT, wild-type; APP, APP/PS1 transgenic mice mutation; CD, control diet; NED, nut-enriched diet.

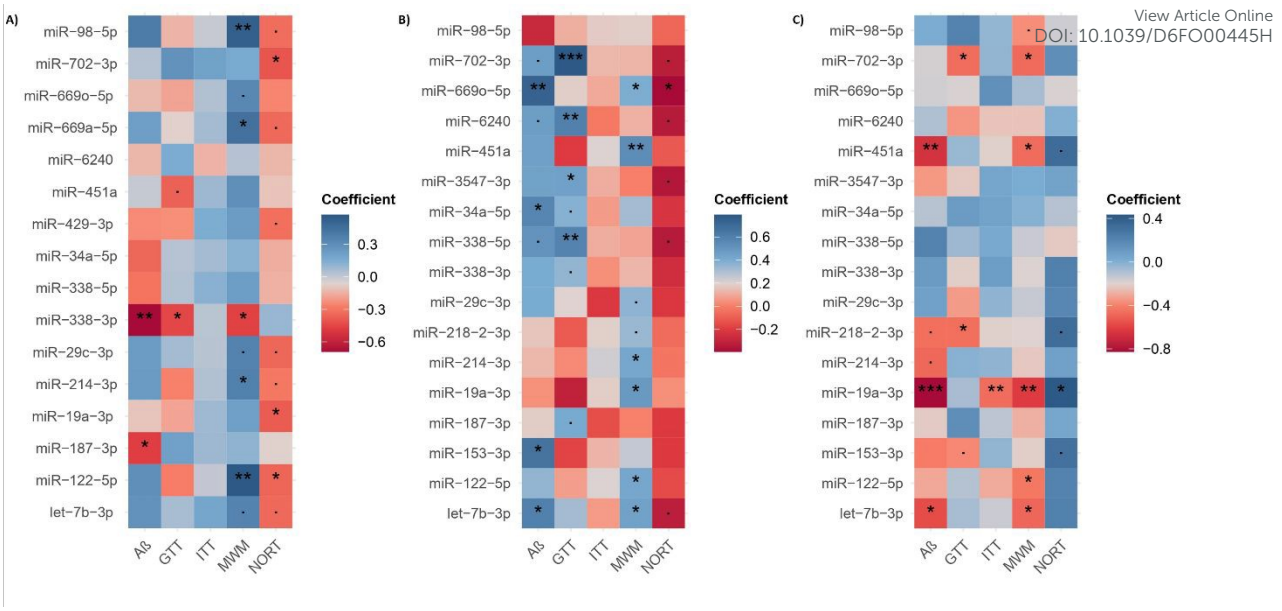

Figure 7. Correlation plot of miRNA expression in serum (A), cortex (B) and hippocampus (C) with amyloid-β concentration in brain (pg of Aβ/µg of protein), GTT, ITT, MWM performance (learning curve AUC) and NORT discrimination index. Correlations were assessed by Pearson or Spearman correlations according to the normality of the data distribution (· p-value < 0.1, * p-value <0.05, ** p-value < 0.01). Abbreviations: Aβ, amyloid β; GTT, glucose tolerance test; ITT, insulin tolerance test; MWM, morris water maze test; NORT, novel object recognition test.

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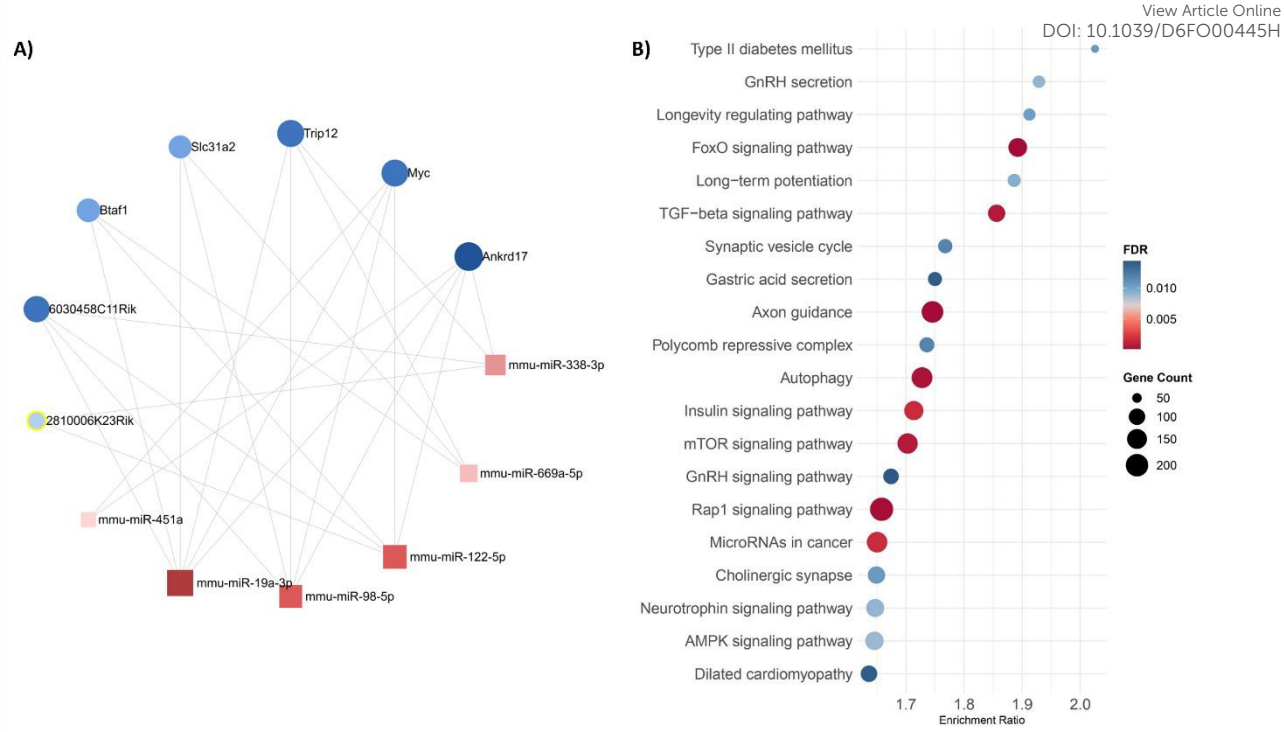

Figure 8. Significantly modulated microRNA functional enrichment analysis. (A) Minimal network of 6 significantly modulated miRNAs and potential target genes; miRNAs are represented in red squares and target genes in blue dots; the size indicated the number of relations. (B) Significant KEGG pathways enrichment analysis of the predicted target genes of the 6 significantly modulated miRNA.

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Nut consumption as a therapeutic strategy to preserve brain function, attenuate neuropathology, and modulate cross-tissue microRNAs in a mouse model of Alzheimer's disease

Availability of data and materials

Data, including raw or processed metadata, software and code, will be fully available upon request to authors.