

A comprehensive molecular study of hsa-miR-140-5p and hsa-miR-31-5p and their target genes *MMP9*, *TNF-α*, *TGFB1*, and *SCN2A* in patients with comorbid Alzheimer's disease and epilepsy

Saghar Zonnar¹, Jafar Amani^{2*}, Iman Salahshourifar¹

¹ Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

² Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Objective(s): Alzheimer's disease (AD) and epilepsy show significant bidirectional clinical comorbidity, yet their shared peripheral molecular mechanisms remain poorly characterized. This study investigated potential dysregulation of hsa-miR-140-5p and hsa-miR-31-5p, along with their target genes (*MMP9*, *TNF-α*, *TGFB1*, *SCN2A*), in patients with comorbid AD and epilepsy.

Materials and Methods: A multi-tiered study was conducted, combining a systematic bioinformatic meta-analysis of five public GEO datasets (over 500 samples) with experimental validation using qRT-PCR in peripheral blood mononuclear cells (PBMCs) and MALDI-TOF mass spectrometry in plasma from 20 comorbid patients and 20 age- and sex-matched healthy controls.

Results: Bioinformatic screening identified a prominent inflammatory network. Experimental validation revealed significant up-regulation of *MMP9* (3.92-fold, $P<0.0001$) and *TNF-α* (2.21-fold, $P=0.023$) in comorbid patients. Conversely, hsa-miR-140-5p, a predicted repressor of *MMP9*, was significantly down-regulated (0.45-fold, $P=0.008$), demonstrating a strong inverse correlation with *MMP9*. Paradoxically, hsa-miR-31-5p was significantly up-regulated (2.8-fold, $P<0.001$). Changes in *TGFB1* and *SCN2A* expression were not statistically significant. Proteomic profiling via MALDI-TOF identified 57 differentially abundant peptide peaks, confirming exclusive detection of Aβ1-42, the loss of neurogranin-derived peptides (absent in patients), and elevated *MMP9* proteolytic fragments in the patient group.

Conclusion: These preliminary findings indicate a shared peripheral molecular signature involving hsa-miR-140-5p, hsa-miR-31-5p, *MMP9*, and *TNF-α*. This highlights neuroinflammatory pathways as shared features of comorbid AD and epilepsy, warranting further evaluation in larger longitudinal cohorts.

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Introduction

Alzheimer's disease (AD) is the leading cause of progressive dementia, marked by extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. Its pathology arises from interacting factors including genetic susceptibility, aging, oxidative stress, mitochondrial dysfunction, and chronic neuroinflammation (1, 2). The amyloid cascade hypothesis proposes that an imbalance between Aβ production and clearance, particularly of Aβ42, triggers downstream tau pathology, synaptic loss, and neuronal death (3). Epilepsy, in contrast, is defined by neuronal hyperexcitability characterized by a predisposition to recurrent unprovoked seizures caused by abnormal, excessive, or synchronous neuronal activity (4). Temporal lobe epilepsy (TLE), the most common focal form, is often associated with hippocampal sclerosis and can produce

cognitive and memory impairments that overlap clinically with early AD (5).

Over the past two decades, the clinical boundaries between AD and epilepsy have become increasingly blurred. Individuals with AD have a six- to ten-fold increased risk of developing unprovoked seizures compared to age-matched controls (6). Conversely, patients presenting with new-onset epilepsy in later life often exhibit biomarkers of AD pathology, such as reduced cerebrospinal fluid (CSF) Aβ42 and elevated tau levels (7). This strong bidirectional association suggests shared fundamental pathogenic mechanisms.

One compelling shared pathway is chronic neuroinflammation. In AD, microglial and astrocytic activation by Aβ aggregates leads to sustained production of pro-inflammatory cytokines, including *TNF-α* and *IL-1β*, thereby exacerbating Aβ production, promoting tau

*Corresponding author: Jafar Amani. Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Vanak Sq. Molasadra St, P.O. Box 19395-5487, Tehran, Iran. Email: jafar.amani@gmail.com, jamani5@bmsu.ac.ir



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Table 1. Demographic and clinical characteristics of study participants

Clinical characteristic	Comorbid patients (n=20)	Healthy controls (n=20)
Age (Years, Median (IQR))	72.5 (68.0–78.0)	71.0 (67.5–76.0)
Gender (Male/Female)	9 / 11	10 / 10
MMSE Score (Median (IQR))	18.5 (15.0–22.0)	29.0 (28.0–30.0)
Epilepsy Type	Focal Temporal Lobe (n=16), Generalized (n=4)	N/A
Median Seizure Frequency	1–2 per month	0
Concomitant Medications	Levetiracetam (n=14), Donepezil (n=12), None (n=0)	None (unmedicated)

phosphorylation, and impairing synaptic function (8). In epilepsy, a similar inflammatory milieu modulates the expression of glutamate and GABA receptors, lowering seizure thresholds and promoting epileptogenesis (9). A key downstream effector is matrix metalloproteinase 9 (MMP9), which degrades extracellular matrix components and tight junction proteins. In AD, blood-brain barrier (BBB) disruption facilitates the influx of neurotoxic plasma proteins; in epilepsy, MMP9-mediated degradation destabilizes perineuronal nets and potentiates excitotoxic injury (10, 11). At the epigenetic level, microRNAs (miRNAs) have emerged as master regulators of these pathogenic processes. These small non-coding RNAs (~22 nucleotides) fine-tune gene expression post-transcriptionally by binding to the 3' untranslated region (3'UTR) of target mRNAs (12). hsa-miR-140-5p is a brain-enriched miRNA that directly targets and represses *MMP9* mRNA (13). Its down-regulation has been reported in AD. hsa-miR-31-5p regulates inflammatory signaling, with documented interactions with *TNF- α* and other cytokines, often induced by NF- κ B inflammatory stimuli (14).

This pilot exploratory study was designed to test the hypothesis that a dysregulated axis involving hsa-miR-140-5p/hsa-miR-31-5p and their targets *MMP9*, *TNF- α* , *ICAM-1*, and *SCN2A* constitutes a shared molecular signature in the comorbidity of AD and epilepsy.

Materials and Methods

Microarray data collection and preprocessing

We consciously included both central (post-mortem brain) and peripheral (blood/serum) datasets to establish an integrative network capable of mapping core pathogenic pathways that cross the blood-brain barrier interface. Rather than evaluating raw baseline expression levels, the analysis focused on the directionality of highly conserved regulatory networks shared between the two compartments. The detailed clinical cohort characterization of patients is described in Table 1.

A systematic search was conducted in the NCBI Gene Expression Omnibus (GEO) database using the keywords “Alzheimer’s disease,” “epilepsy,” “mRNA,” and “miRNA.” Datasets were included if they met: (1) a minimum sample size of 20 per group, (2) a high-reliability microarray

platform (Affymetrix or Illumina), and (3) public availability of raw data files.

The final dataset selection included: for mRNA profiling, GSE5281 (AD, post-mortem brain, n=161), GSE4757 (AD, post-mortem brain, n=142), and GSE209630 (epilepsy, blood, n=68); for miRNA profiling, GSE205661 (AD, serum, n=48) and GSE16759 (epilepsy, blood, n=32). The clinical and technical characteristics of these datasets are detailed in Table 2.

All raw data files were processed in R (version 4.2.1) using Bioconductor packages. For Affymetrix datasets, background correction, quantile normalization, and summarization were performed using the Robust Multi-Array Average (RMA) algorithm implemented in the *oligo* package (15). Illumina data were normalized using the *limma* package’s *normalizeBetweenArrays* function with the quantile method (16). Batch effects were removed using the *ComBat* function from the *SVA* package, with individual GEO dataset accession numbers assigned as distinct batch covariates. Biological group configurations (Disease vs Control) were explicitly protected using the *mod* model matrix design parameter to ensure biological signals were preserved. Differentially expressed genes (DEGs) and miRNAs (DEMs) were identified using linear models with empirical Bayes moderation (LIMMA) (16). Features were deemed significant if they met thresholds of $\log_2$ (Fold Change) ≥ 1 and adjusted *P*-value (Benjamini-Hochberg false discovery rate) < 0.05 . To address tissue heterogeneity, *post hoc* stratified subanalyses were performed separately for blood-only and brain-only datasets. The core genes of interest (*TNF- α* and *MMP9*) retained high significance scores within both strata.

Target prediction and network construction

To establish a functional link between DEMs and DEGs, we used the multiMiR R package to predict target genes (17). This package integrates predictions from 14 major databases, including miRTarBase, TargetScan, miRanda, and DIANA-microT. Only experimentally validated interactions or those predicted by at least three independent databases were retained.

The intersection between predicted miRNA targets and DEGs was identified. A protein-protein interaction (PPI)

Table 2. Characteristics of the gene expression omnibus (GEO) datasets used in the bioinformatic meta-analysis

GEO accession	Platform	GPL ID	Disease	Tissue/Source	Total samples	Case samples	Control samples
GSE5281	Affymetrix HG-U133 Plus 2.0	GPL570	Alzheimer’s disease	Post-mortem brain (various regions)	161	129	32
GSE4757	Affymetrix HG-U133 Plus 2.0	GPL570	Alzheimer’s disease	Post-mortem brain (hippocampus, entorhinal cortex)	142	104	38
GSE209630	Affymetrix Clariom S Human	GPL23038	Epilepsy	Peripheral blood	68	37	31
GSE205661	Affymetrix GeneChip miRNA 4.0	GPL13534	Alzheimer’s disease	Serum	48	24	24
GSE16759	Affymetrix GeneChip miRNA 1.0	GPL8757	Epilepsy	Peripheral blood	32	16	16

network was constructed using the STRING database (version 11.5; confidence score threshold 0.9) (18). The network was imported into Cytoscape (version 3.9.1), and the top 10 hub genes were identified using the CytoHubba plugin based on Degree, Closeness, and Betweenness centrality measures.

Clinical cohort and sample collection

All procedures were approved by the Institutional Review Board and adhered to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians. The patient group consisted of 20 individuals with confirmed clinical diagnoses of both AD and epilepsy. AD diagnosis was established according to NIA-AA criteria (19), supported by MMSE score < 24 and MRI showing hippocampal atrophy. Epilepsy was diagnosed according to ILAE definition (20), requiring at least two unprovoked seizures with video-EEG confirmation.

Patients with active systemic infections, documented autoimmune flare-ups, active malignancies, or status epilepticus hospitalizations within 4 weeks before blood sampling were strictly excluded from participation. Controls had no history of neurological or psychiatric disorders, seizures, cognitive impairment (MMSE > 28), or first-degree family history of AD or epilepsy. Peripheral blood (5 ml) was drawn into EDTA tubes and processed within two hours.

RNA extraction, quality control, and cDNA synthesis

Total RNA, including small RNAs, was extracted from PBMCs using TRIzol® Reagent (Invitrogen, USA) (21). RNA was further purified and DNase-treated using the RNeasy Mini Kit (Qiagen, Germany). Concentration and purity were determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). High-quality RNA was defined by A260/A280 ratio between 1.8–2.1 and A260/A230 ratio > 2.0. RNA integrity was confirmed by visualizing 18S and 28S rRNA bands on a 2% agarose gel. All samples that met the quality criteria were stored at -80 °C.

For cDNA synthesis, two separate reactions were performed to accommodate both mRNA and miRNA analyses. For mRNA targets (MMP9, TNF- α , TGF β 1, SCN2A, and the housekeeping gene GAPDH), 500 ng of total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with a blend of oligo(dT) and random hexamer primers. For miRNA analysis (hsa-miR-140-5p, hsa-miR-31-5p, and the endogenous control U6 snRNA), 500 ng of total RNA was

reverse-transcribed using the miScript II RT Kit (Qiagen, Germany), which uses a universal reverse transcription primer that adds a poly-A tail and a universal tag sequence to all miRNAs in the sample. All cDNA samples were stored at -20 °C before qRT-PCR.

Quantitative real-time PCR (qRT-PCR)

Gene-specific primers for the mRNA targets and the housekeeping gene were designed with the NCBI Primer-BLAST tool to span exon-exon junctions, thereby avoiding amplification of any potential genomic DNA contaminants. Primer sequences, amplicon sizes, and thermocycling conditions are listed in Table 3. For miRNA quantification, pre-designed, ready-to-use miScript Primer Assays (Qiagen, Germany) were used for hsa-miR-140-5p (MS00003536), hsa-miR-31-5p (MS00003274), and U6 snRNA (MS00033705).

Custom qPCR primer amplification efficiencies were verified using 5-point standard serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32), yielding linear efficiencies ranging between 96% and 103% with clear single-peak dissociation melt profiles for all targets. The expression values of the reference genes GAPDH and U6 snRNA showed high stability indices (M-values < 0.5) across both patient and control subsets, as determined by the geNorm algorithm, confirming their validity as reliable endogenous normalizers in this cohort. qRT-PCR was performed in triplicate on a Rotor-Gene Q cycler (Qiagen). Each 20 μ L reaction contained 10 μ L of 2x SYBR Green Master Mix, 2 μ L of cDNA (diluted 1:5), 2 μ L of primer mix, and 6 μ L of RNase-free water. Thermocycling profile: 95 °C for 15 min; 40 cycles of 94 °C for 15 sec, 60 °C for 30 sec, and 70 °C (miRNA) or 72 °C (mRNA) for 30 sec. Melting curve analysis confirmed single-product amplification. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (22), with GAPDH and U6 snRNA as internal controls.

MALDI-TOF mass spectrometry

To provide orthogonal validation of our transcriptomic findings at the proteomic level, we performed a comprehensive peptide profiling of plasma samples using MALDI-TOF mass spectrometry. Plasma was first depleted of the six most abundant high-abundance proteins (albumin, IgG, IgA, transferrin, haptoglobin, and alpha-1-antitrypsin) using the Agilent Human 6 Multiple Affinity Removal System (MARS) column to enhance the detection of lower-abundance peptides. The depleted plasma was

Table 3. Primer sequences and thermocycling parameters for qRT-PCR

Gene	Primer sequence (5' $\rightarrow$ 3')	Amplicon size (bp)	Annealing temp (°C)
MMP9	Forward: GCCACTACTGTGCCTTTGAGTC	163	60
	Reverse: CCCTCAGAGAATCGCCAGTACT		
TNF- α	Forward: CTCTTCTGCCTGCTGCACTTTG	108	60
	Reverse: ATGGGCTACAGGCTTGTCACCT		
TGF β 1	Forward: GCGCTGCTTGCAGAGATTAA	122	60
	Reverse: GGCCAGGTGCTTGTCTAGCA		
SCN2A	Forward: AGGTGGCTGAGGAATGTTTC	140	60
	Reverse: CCGTGTTTCATCATCCATGTG		
GAPDH	Forward: GTCTCCTCTGACTTCAACAGCG	121	60
	Reverse: ACCACCTGTGTGCTGTAGCCA		

Table 4. Top differentially expressed genes (DEGs) from the integrated bioinformatic analysis

Gene symbol	log ₂ (fold change)	Adjusted P-value	Gene symbol	log ₂ (fold change)	Adjusted P-value
Top up-regulated Genes			Top down-regulated genes		
TNF-α	+3.04	2.25E-18	SCN2A	-4.67	2.43E-14
CXCR4	+2.59	4.50E-23	CD44	-4.12	2.20E-16
MMP9	+2.19	1.57E-14	NDUFA7	-4.00	6.43E-15
IL6	+1.82	6.96E-16	CASP1	-3.99	2.06E-27
VEGFA	+1.14	7.78E-06	MAPK14	-2.99	6.76E-12
TGFB1*	+3.73	1.67E-16	FLT3	-2.74	2.30E-21
IFNG	+1.18	3.80E-15	TNFSF13B	-2.68	1.69E-23
IL8	+1.54	0.000578	TGFB1*	-2.46	2.90E-14
CCL20	+1.01	2.07E-05	IL1RAP	-2.45	2.10E-11
CSF1	+1.01	0.000131	CCR2	-2.34	1.22E-10

Note: TGFB1 appears in both lists due to its opposite regulation in AD (down) versus epilepsy (up) datasets.

then concentrated and digested with sequencing-grade trypsin (1:50 enzyme-to-protein ratio) at 37 °C for 16 hr. The resulting peptide mixture was separated by nano-liquid chromatography (nano-LC) on an EASY-nLC 1200 system (Thermo Fisher Scientific) using a 50 cm C18 reversed-phase column (Acclaim PepMap 100, 75 µm inner diameter). Peptides were eluted with a linear gradient of 2–35% acetonitrile in 0.1% formic acid over 120 min at a flow rate of 300 nL/min. The eluent was automatically spotted onto a 1536-spot Bruker AnchorChip target plate using a Probot micro-fraction collector (Agilent), with one fraction collected every 10 sec. The matrix, α-cyano-4-hydroxycinnamic acid (CHCA) at a concentration of 5 mg/ml in 50% acetonitrile/0.1% trifluoroacetic acid, was then applied to the target plate using an automated sprayer (HTX Technologies).

The target plate was analyzed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in positive ion reflector mode (m/z 700–3500). Instrument calibration was performed daily using peptide calibration standards. For each spot, 500 laser shots were accumulated in a raster pattern. Raw mass spectra were processed using FlexAnalysis 3.4 for baseline subtraction and peak picking (signal-to-noise ratio threshold ≥ 5). Peak lists were exported to ClinProTools 3.0 for statistical analysis. A two-sample t-test ($P < 0.05$) identified differentially expressed peaks. Principal component analysis (PCA) visualized group separation. For peptide identification, MS/MS fragmentation was performed on differentially expressed peaks. Fragment ion spectra were searched against the UniProt human proteome database (taxonomy ID: 9606) using Mascot 2.8 (Matrix Science) with a precursor mass tolerance of 0.2 Da and a fragment mass tolerance of 0.3 Da. Mascot score thresholds were set at >40 for high-confidence identity; peptide-level FDR was set at $<1\%$. Sample processing was conducted using randomized sample batches with duplicate technical injections.

Statistical analysis

Statistical analyses for qRT-PCR and MALDI-TOF data were performed using SPSS version 22 (IBM). Normality was assessed using the Shapiro-Wilk test. Since the sample size in each group was <30 , non-parametric tests were used. The Mann-Whitney U test was used to compare relative expression levels between the patient and control groups. Data are presented as median [interquartile range, IQR] to align with non-parametric analysis. A P -value < 0.05 was considered statistically significant.

Results

Bioinformatic identification of differentially expressed molecules

Our integrated bioinformatic analysis of the five GEO datasets yielded a substantial number of differentially expressed features. We identified 109 DEGs consistently altered across the AD and epilepsy datasets. Of these, 62 were up-regulated, and 47 were down-regulated. In parallel, we identified 45 DEMs, with 28 being up-regulated and 17 down-regulated. The complete lists of the top 50 up- and down-regulated DEGs are provided in Table 4.

Key DEGs of interest included MMP9 ($\log_2FC = +2.59$ in the combined analysis), TNF-α ($\log_2FC = +3.04$), and SCN2A ($\log_2FC = -4.67$). Notably, TGFB1 exhibited a complex, disease-specific regulation, being down-regulated in AD datasets ($\log_2FC = -2.46$) but up-regulated in the epilepsy dataset ($\log_2FC = +3.73$), highlighting its context-dependent role. Among the DEMs, two miRNAs of primary interest were hsa-miR-140-5p, which was consistently down-regulated across datasets ($\log_2FC = -1.21$, $P = 0.0258$), and hsa-miR-31-5p, which showed a more nuanced pattern (down-regulated in AD, $\log_2FC = -2.19$, but up-regulated in epilepsy, $\log_2FC = +1.45$).

Hub gene and miRNA-mRNA regulatory network analysis

Intersection of predicted DEM targets and DEGs yielded 64 genes. PPI network construction (62 nodes, 581 edges) revealed high functional interconnectivity. CytoHubba identified top 10 hub genes: TNF-α, IL6, TGFB1, VEGFA, SCN2A, MMP9, TLR1, CD44, CASP1, and CD68 (Table 5). This list supports the central role of inflammatory signaling (TNF-α, IL6, TGFB1, TLR1, CD68), extracellular matrix remodeling (MMP9, VEGFA, CD44), and neuronal excitability (SCN2A, CASP1). The miRNA-mRNA network confirmed interactions of interest: hsa-miR-140-5p targeting MMP9, and hsa-miR-31-5p targeting TNF-α.

Experimental validation by qRT-PCR

qRT-PCR validated bioinformatic predictions (Figure 1, Table 6). MMP9 expression was significantly up-regulated in patients (median fold-change: 3.92, $P < 0.0001$). TNF-α was significantly elevated (median fold-change: 2.21, $P = 0.023$). TGFB1 and SCN2A were down-regulated (2.21-fold and 3.01-fold, respectively) but did not reach statistical significance ($P = 0.22$ and $P = 0.31$, respectively).

At the miRNA level, hsa-miR-140-5p was significantly down-regulated (0.45-fold, $P = 0.008$), consistent with MMP9 up-regulation. A strong inverse correlation was

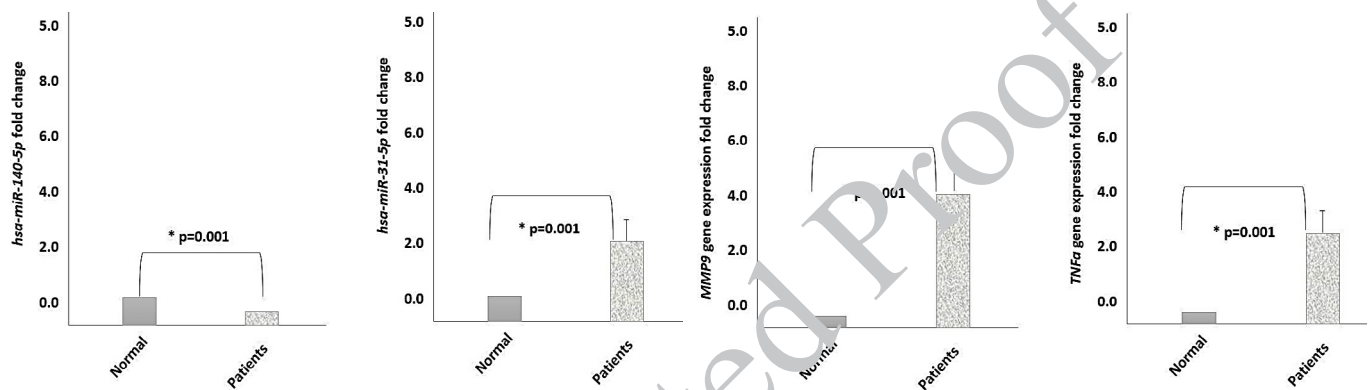
Table 5. Top 10 hub genes identified from the protein-protein interaction (PPI) network

Rank	Gene symbol	Degree	Closeness	Betweenness	Description
1	TNF- α	50	0.078	125.4	Tumor necrosis factor alpha; master pro-inflammatory cytokine
2	IL6	49	0.077	120.1	Interleukin 6; pleiotropic cytokine with pro- and anti-inflammatory roles
3	TGFB1	42	0.075	105.8	Transforming growth factor beta 1; a key immunosuppressive cytokine
4	VEGFA	39	0.073	98.2	Vascular endothelial growth factor A; regulates angiogenesis and vascular permeability
5	SCN2A	34	0.070	85.6	Sodium voltage-gated channel alpha subunit 2; critical for neuronal action potential initiation
6	MMP9	32	0.069	80.3	Matrix metalloproteinase 9; degrades extracellular matrix and tight junctions
7	TLR1	30	0.068	75.0	Toll-like receptor 1; innate immune sensor for bacterial components
8	CD44	28	0.066	68.9	CD44 molecule; adhesion molecule involved in cell migration and lymphocyte homing
9	CASP1	25	0.064	60.7	Caspase 1; key enzyme in the inflammasome complex and pyroptosis
10	CD68	24	0.063	58.1	CD68 molecule; a marker for activated/macrophages/microglia

observed between hsa-miR-140-5p and MMP9 (Spearman's $\rho = -0.62$, $P = 0.004$). Paradoxically, hsa-miR-31-5p was significantly up-regulated (2.8-fold, $P < 0.001$), with a positive correlation to TNF- α (Spearman's $\rho = +0.58$, $P = 0.007$), suggesting a more complex regulatory relationship.

MALDI-TOF proteomic profiling

MALDI-TOF on plasma from 10 patients and 10 controls detected >250 distinct m/z peaks. Statistical comparison (t-test, $P < 0.05$) identified 57 significantly differentially abundant peptides (Table 7).

**Figure 1.** Results of selected hub genes and miRNAs expression**Table 6.** qRT-PCR results: Relative expression of target genes and miRNAs in Alzheimer's patient vs control groups

Target molecule	Fold change (patients vs. controls)	P-value	Mean ΔCt (patients)	Mean ΔCt (controls)
MMP9	3.92	<0.0001	-1.65	-3.17
TNF- α	2.21	0.023	-0.58	-1.47
TGFB1	2.21	0.22	1.05	0.16
SCN2A	-3.01	0.31	1.52	0.30
hsa-miR-140-5p	0.45	0.008	3.85	2.15
hsa-miR-31-5p	2.80	<0.001	0.95	2.30

Table 7. Key differentially expressed peptides identified by MALDI-TOF proteomic profiling

m/z	Putative identity	Parent protein (UniProtID)	Fold change	P-value	Biological significance
2145.9	A β 1-42	APP (P05067)	$+\infty$ (Patient-specific)	0.004	Core component of amyloid plaques in AD
1867.4	Neurogranin (48-64)	NRGN (Q92849)	-100% (Absent in patients)	0.001	Postsynaptic protein; loss correlates with synaptic dysfunction in AD and epilepsy
1372.1	MMP9 (391-402)	MMP9 (P14780)	+1.9	0.023	Direct evidence of elevated MMP9 proteolytic activity
1046.5	Clusterin Fragment	CLU (P10909)	+2.3	0.002	Chaperone protein involved in A β clearance and BBB integrity
987.5	STXB1 Fragment	STXB1 (Q969S8)	-1.6	0.011	Regulates synaptic vesicle release; mutations cause epileptic encephalopathy

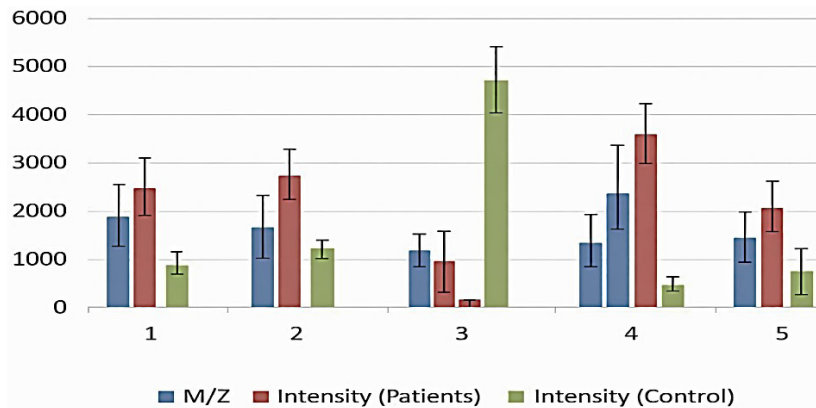


Figure 2. Comparison of Peptide Peak Profiles Between the Studied Groups Based on m/z (Mass-to-Charge Ratio)

Key findings: A β 1-42 was detected above the instrumentation's sensitivity threshold exclusively in the patient cohort. Neurogranin-derived peptide (48-64) showed near-total loss in patients (absent or below the detection limit). MMP9-derived peptide (391-402) was elevated 1.9-fold ($P=0.023$). Clusterin fragment was elevated 2.3-fold ($P=0.002$). STXBP1 fragment was reduced 1.6-fold ($P=0.011$).

PCA of the entire peptide profile effectively differentiated between groups, with PC1 accounting for 72% of total variance and showing complete separation (Figure 2).

Discussion

This pilot exploratory study provides a multi-layered molecular portrait of comorbid AD and epilepsy pathogenesis. By integrating bioinformatic dissection of public datasets with experimental validation and orthogonal proteomic confirmation, we identified a distinct peripheral molecular pattern that co-occurs with alterations in specific microRNAs and their inflammatory- and neuroplasticity-related target genes.

Our most robust finding is the significant up-regulation of MMP9 at both mRNA and proteomic levels. MMP9 is a critical effector of neuroinflammation; its overexpression compromises BBB integrity by degrading basal lamina and tight junction components (23). This facilitates the influx of neurotoxic plasma proteins while hindering A β efflux. In AD, this accelerates amyloid plaque formation and neuronal injury; in epilepsy, BBB disruption contributes to epileptogenesis through ionic imbalance and albumin extravasation (24). The observed down-regulation of hsa-miR-140-5p, a known repressor of MMP9, provides an associative mechanistic link. The strong inverse correlation between these molecules supports an observed molecular association consistent with previous studies in other inflammatory models (25).

The significant elevation of TNF- α further supports the role of chronic neuroinflammation. TNF- α modulates synaptic function by increasing AMPA receptor surface expression and decreasing GABA-A receptor expression, creating a state of neuronal hyperexcitability conducive to seizures (26). In AD, chronic TNF- α signaling drives microglial activation and promotes tau phosphorylation.

The concurrent up-regulation of both hsa-miR-31-5p and TNF- α represents a statistical correlation rather than proof of a direct functional interaction. *In silico* databases suggest targeting sequences, but our parallel

clinical up-regulation suggests that hsa-miR-31-5p expression could be downstream of chronic inflammatory feedback networks, such as NF- κ B signaling. The NF- κ B transcription factor can activate miRNA transcription, including miR-31 (27). Therefore, the observed up-regulation may represent a secondary consequence of ongoing inflammation, a hypothesis that warrants further mechanistic validation via luciferase reporter assays in future functional studies. Alternative explanations include shifts in cell type proportions among circulating leukocytes and varying systemic inflammatory kinetics during seizure-free intervals. The lack of statistically significant changes in TGFB1 and SCN2A warrants careful interpretation. TGFB1 is a pleiotropic cytokine with dual roles in neurodegeneration: neuroprotective in early phases but potentially contributing to astrocytic scarring and amyloidogenesis in chronic stages (28). The opposing regulation observed in bioinformatic data (down in AD brain, up in epilepsy blood) suggests its net peripheral effect may be balanced or stage-dependent. Regarding SCN2A, while loss-of-function mutations cause severe epileptic encephalopathies, its role in acquired late-onset epilepsy with comorbid AD is less clear (29). Moreover, our peripheral blood analysis may not adequately reflect neuronal-specific changes, which could be primarily post-translational or restricted to specific brain populations. The MALDI-TOF results serve as orthogonal validation. The detection of A β 1-42 exclusively in patients is a biochemical indicator of AD pathology. The loss of neurogranin-derived peptide is noteworthy, as neurogranin is essential for synaptic plasticity and long-term potentiation (30). Its loss correlates with synaptic failure underlying cognitive impairment in both AD and epilepsy (31). The identification of an MMP9-derived peptide provides direct evidence that mRNA up-regulation translates to increased proteolytic activity.

This study has several limitations. First, our reliance on peripheral blood as a proxy for CNS pathology: the brain and blood are connected but not identical. Future studies should validate findings in CSF. Second, our sample size ($n=20$ for qRT-PCR, $n=10$ for MALDI-TOF), while achieving >80% power for large effects, is modest and may have limited detection of subtle changes. Third, the cross-tissue heterogeneity in our bioinformatic meta-analysis (post-mortem brain vs peripheral blood) introduces biological variability. While our stratified sub-analyses confirmed the significance of core genes in both tissue types, direct cross-tissue comparability remains a limitation. Fourth, this is a correlative study establishing associations, not causation.

Future work using cellular and animal models is needed to test causal relationships functionally. Fifth, the study lacks an independent external validation cohort, a critical limitation that must be addressed before any clinical utility of the biomarker can be established.

Conclusion

This pilot exploratory study identified a peripheral molecular signature comprising hsa-miR-140-5p, hsa-miR-31-5p, MMP9, and TNF- α in patients with comorbid AD and epilepsy. Although these associations provide conceptual insights into shared neuroinflammatory pathways, therapeutic strategies targeting these pathways remain speculative and require extensive functional validation in animal models before clinical consideration.

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Authors' Contributions

S Z designed and performed the experiments and collected data; J A discussed the results and strategy, supervised, directed, and managed the study; IS gave final approval of the version to be published.

Conflicts of Interest

The authors declare that they have no financial conflicts of interest.

Declaration

We acknowledge the use of artificial intelligence (DeepSeek) as a writing assistant in this article. All AI-generated modifications were reviewed and edited by our team to ensure accuracy and coherence.

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