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ORIGINAL RESEARCH

# Altered Neuroplasticity in Epilepsy is Associated with Neuroinflammation and Oxidative Stress: In vivo Evidence of Brain-Derived Extracellular Vesicles

Shun Wang[1,*], Haiju Zhang[1,*], Ruiling Li[2], Zhongchun Liu[1], Dan Xiang[2]

[1]Department of Pediatrics, Renmin Hospital of Wuhan University, Wuhan, Hubei, People's Republic of China; [2]Department of Psychiatry, Renmin Hospital of Wuhan University, Wuhan, Hubei, People's Republic of China

*These authors contributed equally to this work

Correspondence: Dan Xiang; Zhongchun Liu, Department of Psychiatry, Renmin Hospital of Wuhan University, No. 99 Jiefang Road, Wuchang District, Wuhan, Hubei, 430060, People's Republic of China, Email xiangdannuli@163.com; zcliu6@whu.edu.cn

**Purpose:** Recurrent seizures lead to self-reconstruction of the central nervous system, which is termed the neuroplasticity of epilepsy. While preclinical studies implicate neuroinflammation and oxidative stress in epilepsy-associated neuroplasticity, in vivo molecular-level evidence in humans is lacking.

**Patients and Methods:** We used astrocyte-derived extracellular vesicles (ADEVs) and neuron-derived extracellular vesicles (NDEVs) as brain-derived biomarkers to explore biomarkers of neuroplasticity, neuroinflammation, and oxidative stress. A total of 50 patients in the epilepsy group (EP) and 25 matched healthy controls (HC) were recruited for this study. Plasma ADEVs and NDEVs were isolated and confirmed, and the levels of the EV marker CD81, the neuroplasticity marker brain-derived neurotrophic factor (BDNF), and the neuroinflammation marker tumor necrosis factor α (TNF-α) in ADEVs, as well as the markers of oxidative stress, superoxide dismutase 1 (SOD1) and malondialdehyde (MDA), in NDEVs were measured.

**Results:** BDNF levels in ADEVs and SOD1 levels in NDEVs from EP were significantly lower than those in HC, whereas TNF-α levels in ADEVs and MDA levels in NDEVs were significantly increased, and the results remained stable after normalization by CD81. Spearman correlation analysis revealed that BDNF levels in ADEVs were negatively correlated with TNF-α levels in ADEVs and MDA levels in NDEVs and positively correlated with SOD1 levels in NDEVs.

**Conclusion:** The innovative use of ADEVs and NDEVs as brain-derived biomarkers in this study provides in vivo evidence that epilepsy may result in impaired neuroplasticity and may be associated with increased neuroinflammation and oxidative stress.

**Keywords:** epilepsy, extracellular vesicles, neuroplasticity, neuroinflammation, oxidative stress

## Introduction

Epilepsy is a common neurological disorder caused by abnormal synchronous discharges of neurons in the brain and is characterized by recurrent, spontaneous, and stereotyped seizures. Epilepsy is often associated with comorbidities and reflects a significant disease burden that negatively affects patient quality of life.[1] Despite the variety of available drug therapies, nearly 1/3 of patients with epilepsy are resistant to antiepileptic drugs (AEDs) and develop drug-refractory epilepsy.[2] Our understanding of the pathological alterations associated with epilepsy remains limited, underscoring the necessity for further investigation.

Recurrent epileptic seizures resulting from abnormal synchronous neuronal discharges lead to self-reconstruction of the central nervous system, which is termed the neuroplasticity of epilepsy.[3] Astrocytes participate in the regulation of neuroplasticity[4] and the BDNF produced by astrocytes is an endogenous neurotrophic factor, which is crucial for promoting neurogenesis, neural differentiation, and synaptic plasticity.[5] Moreover, it can serve as a reliable biomarker of changes in neuroplasticity of epilepsy.[6] Previous in vivo studies on the neuroplasticity marker BDNF in patients with epilepsy have focused mainly on peripheral blood, with few studies on cerebrospinal fluid, and the results have been

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inconsistent. Some studies have found higher serum BDNF in patients with epilepsy,[7] even associated with more severe conditions of primary epilepsy.[8] In contrast, other studies have shown that BDNF levels are decreased in patients with epilepsy.[9,10] Nowroozi et al's meta-analysis found no significant overall difference in BDNF levels between epileptic and control subjects, though partial patients with epilepsy had lower BDNF levels than controls—a trend absent in generalized epilepsy.[8] Therefore, given the inconsistency of research findings, it is necessary to conduct more studies using more effective methods to evaluate changes in in vivo neuroplasticity biomarkers in patients with epilepsy.

Oxidative stress and neuroinflammation, which can result from recurrent seizures, may be potential contributors to neuroplasticity in epilepsy.[11] Studies reported that the serum levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) are significantly lower in drug-resistant epilepsy patients than in healthy controls, whereas malondialdehyde (MDA) levels, a lipid product reflecting oxidative stress, were significantly higher,[12] accompanied by upregulation of pro-inflammatory factors such as TNF-α, IL-6 and IL-1β[13] These alterations may synergistically affect neuroplasticity in epilepsy by altering the neuronal microenvironment, interfering with neuroplasticity-related signaling pathways, and affecting the neurotransmitter system.[11,14,15] An antioxidant and metal chelator, curcumin, was able to attenuate gliosis and cytokine levels in pentylenetetrazol-induced epilepsy, exerting a role in modulating neuroplasticity.[16] Other redox- and anti-inflammatory-based therapies, such as coenzyme Q10[17,18] and N-acetylcysteine, have also been shown to have central nervous system antioxidant and anti-inflammatory effects, thereby exerting a role in attenuating seizures and modulating neuroplasticity in animal models to reduce complications.[19] More reliable human in vivo evidence is needed to support these studies suggesting that altered neuroplasticity in epilepsy is associated with oxidative stress and inflammation. Current clinical studies predominantly rely on peripheral biomarkers or invasive detection methods,[11–13,15] whereas brain-derived extracellular vesicles (BDEVs) technology offers a novel non-invasive approach for assessing central oxidative stress and neuroinflammation.[20,21] After crossing the blood–brain barrier, BDEVs can be enriched in the peripheral blood on the basis of surface markers of their source cells.[22] For some target molecules, the correlation coefficient between BDEVs-carried and and CSF-present concentrations exceeds 0.9,[23] suggesting BDEVs can be used as brain-derived biomarkers for in vivo source cells status without brain tissue biopsy or lumbar puncture. A previous study by our laboratory provided reliable in vivo evidence for the neurogenesis hypothesis, neuroinflammation hypothesis and neuroplasticity alterations in depression through the detection of changes in doublecortin (DCX) in plasma NDEVs[24] and in TNF-α and BDNF in plasma ADEVs[25,26] and formed the basis for this study.

The present study used plasma BDEVs as in vivo brain-derived biomarkers to investigate the following:

1. As astrocytes are important players in neuroinflammation[27,28] and are the main BDNF-producing cells,[5] our objective was to assay BDNF and TNF-α levels in plasma ADEVs to assess differences in neuroplasticity and neuroinflammation between patients with epilepsy and healthy controls.
2. Measurement of SOD1 and MDA levels in plasma NDEVs to assess CNS oxidative stress in epilepsy.
3. Correlation between neuroplasticity and CNS oxidative stress and neuroinflammation in epilepsy.

# Methods

## Sample Size Calculation

Given the dearth of studies examining BDEV-carrying contents in patients with epilepsy, we elected to utilize an epilepsy serum BDNF concentration study as a reference for sample size calculation. In this study, the effect size of BDNF was 0.78;[10] thus, the effect size was set to 0.78, the power (1-β) was 0.85, the α was 0.05 (two sided), and the allocation ratio was 2, which resulted in the need for 70 persons (47 in the EP and 23 in the HC). The final sample size was set at 75 (50 in the EP and 25 in the HC) in consideration of other potential contingencies. Sample size was calculated using G*Power (ver. 3.1.9.7).[29]

## Human Subjects

This observational study included 50 children with drug-treated epilepsy who were hospitalized in the pediatric department of Wuhan University Renmin Hospital from January 2022 to November 2023, and 25 children who attended

the Child Health Clinic for health check-ups during the same period were also selected as healthy controls. In accordance with the Declaration of Helsinki, the study was approved by the Ethical Review Committee of Wuhan University People's Hospital.[30] The parents or legal guardians of the patients were informed about the purpose of the study and signed an consent form. The following inclusion criteria were applied: (1) the diagnosis of epilepsy conforms to the definition and classification of epilepsy by the 2017 International League Against Epilepsy[31] aged 6–16 years. The exclusion criteria for patients were as follows: (1) had symptomatic epilepsy; (2) first-episode drug-naive patients; (3) had a history of other organic or systemic diseases; (4) were treated surgically for epilepsy; (5) use of glucocorticoids, immunosuppressants or antioxidants in the last three months.

## Collection of Plasma and Enrichment of NDEVs and ADEVs

All participants fasted for 6 hours and 5 mL blood samples were obtained via established venipuncture procedures. Blood samples taken in ethylenediaminetetraacetic acid (EDTA) polypropylene tubes were centrifuged at 2000 × g for 15 minutes at room temperature within 2 hours to extract plasma.The plasma was dispensed into 600 μL aliquots and stored at −80 °C for later use. On the test day, the plasma was thawed and immediately centrifuged at 2000 × g for 10 minutes to remove all the cell debris. Then, the sample was heated at 10,000 × g for 10 minutes at 4 °C to remove large microvesicles and platelets, and a 500 μL aliquot of preclarified plasma was extracted for later use. EVs were isolated in the following two steps. First, total plasma EVs were extracted from the preclarified plasma via size exclusion chromatography (SEC) (qEVoriginal 35 nm, IZON Science, Christchurch, New Zealand) according to previous methods.[32] Briefly, each column was washed with 20 mL of PBS, and 500 μL of preclarified plasma was placed on the qEVoriginal column and eluted with PBS. In the separation process of each sample, the first 3 mL of the fraction was removed, and the remaining 2.5 mL of the fraction rich in extracellular vesicles was collected. Next, the Amicon Ultra 10kd centrifugal filter device (Merck Life Science, USA) was used to centrifuge at 10000 × g for 10 minutes to obtain the concentrated total EVs. Calcium and magnesium-free Dulbecco's phosphate-buffered saline (DPBS) with protease and phosphatase inhibitor cocktails were added to a total volume of 350 μL per sample for subsequent use. Second, ADEVs and NDEVs were extracted from total extracellular vesicles in the same manner as described in a previous study.[25,33] Briefly, immunoprecipitation was used to target a CD171/L1 cell adhesion molecule (L1CAM) biotinylated antibody (eBio5G3 (5G3), Thermo Fisher Scientific, Catalog: 13-1719-82) bound to a neuronal surface marker and to target a glutamine aspartate transporter (GLAST) biotinylated antibody (Miltenyi Biotec, Auburn, CA, Catalog number: 130-118-984) bound to an astrocyte surface marker to isolate $L1CAM^{+}$EVs and $GLAST^{+}$EVs, respectively (Please refer to the Supplementary Material for details of the second step).

## EV Characterization

### Western Blotting

Western blotting was performed to detect three exosomal markers with a rabbit anti-cluster of differentiation (CD) 63 antibody (Abcam, Catalog# ab134045), a mouse anti-Alix antibody (Proteintech, Catalog# 67715-1-Ig), a rabbit anti-TSG101 antibody (Abcam, Catalog# ab125011), and an anti-human synaptosomal associated protein 25 kDa (SNAP25) (Thermo Fisher Scientific, Catalog# MA5-17610) antibody targeting neurons. An anti-glial acidic protein (GFAP) antibody (Abcam, Catalog# ab68428) was used to target astrocytes.

### Transmission Electron Microscopy (TEM)

TEM scanning was performed on the sample to observe the morphology of the ADEVs and NDEVs. A total of 20 μL of NDEV or ADEV samples were added dropwise onto a 200-mesh grid and incubated at RT for 10 minutes. Then, negative staining with 2% phosphotungstic acid was performed for 3 minutes, and the remaining liquid was removed via filter paper. A JEM-1400 transmission electron microscope (Hitachi High Tech, Tokyo, Japan) was then used to observe the samples.

### Nanoparticle Tracking Analysis (NTA)

Three technical replicates of NTA were analyzed for both ADEVs and NDEVs isolated from the same sample. The EVs sample were diluted 100 times with 1X PBS (Biological Industries, Israel). NTA technology (Particle Metrix, Meerbusch,

Germany) and the ZetaView 8.04.02 software were used to analyze the particle size and concentration distribution of EVs. The following parameters were set for the detection of EVs: sensitivity: 80; width: 100; minimum area: 10 pixels; maximum area: 1000 pixels; and minimum brightness: 30.

### Protein Measurements

The levels of CD81, an EV marker in extracellular vesicles (EVs), were measured via an enzyme-linked immunosorbent assay (ELISA) kit (American Research Products Inc., Catalog# CSB-EL004960HU) according to the manufacturer's instructions. TNFα and BDNF levels in ADEVs and SOD1 and MDA levels in NDEVs were measured via the multifactorial Luminex platform from CloudClone. The detected proteins were normalized according to the CD81 protein concentration in ADEVs and NDEVs by setting the mean CD81 level of each group to 1.00 and normalizing its recovery to the relative value of each sample.[25]

## Statistical Analysis

For the descriptive data analysis, the chi-square test was used for categorical data. Numerical data were first tested for a normal distribution (Shapiro–Wilk test); two-sample t tests were used for normally distributed data, and the Mann–Whitney *U*-test was used for nonnormally distributed numerical data. We used a general linear model (GLM) to compare the differences in target protein concentrations between the two groups, setting group as the independent variable and target protein concentrations as the dependent variable, with age and sex as covariates. Due to inconsistencies in normalization for EVs, we tested the target protein concentration normalized to the CD81 concentration as a sensitivity analysis to obtain a robust result. The model-fitted estimated marginal means (EMMs) and their associated 95% confidence intervals (CIs) were used to describe the differences between the two groups. In additional exploratory analyses, we examined differences in target protein levels between subgroups (focal subgroup vs generalized subgroup, drug-resistant subgroup vs drug-responsive subgroup) via the same methodology, ie, GLM. Spearman's rank correlation analysis was employed to assess associations between target protein concentrations in patients with epilepsy. We used Bonferroni corrections to control the false discovery rate. Statistical analyses were performed using SPSS 26 and visualised in ORIGIN 2021. A two-tailed $P$ value <0.05 was considered to indicate statistical significance.

# Results

## Participant Characteristics

Due to the loss of blood samples from one patient with epilepsy and the noncooperation of three healthy control children during blood collection, the study ultimately enrolled 49 children with epilepsy and 22 healthy control children. Table 1 lists the characteristics of the participants. There were no differences in sex or age between the two groups.

**Table 1** Descriptive Data of Included Participants

| | EP (n=49) | HC (n=22) | $t/\chi^2$ | *P* |
|---|---|---|---|---|
| Sex (male/female) | 25/24 | 10/12 | 0.188 | 0.664 |
| Age (Means±SD) | 9.15±0.44 | 9.59±0.44 | −0.523 | 0.546 |
| Age at onset(years) | 7 (6, 8) | – | – | – |
| Disease duration(years) | 1 (0.5, 2) | – | – | – |
| Seizure type(Focal/Generalized/Unknown onset) | 27/22/0 | – | – | – |
| Drug-resistant epilepsy (Yes/No) | 11/38 | | | |

**Abbreviations**: EP, epilepsy; HC, health control; SD, standard deviation.

## Confirmation of ADEVs and NDEVs

The ADEVs and NDEVs obtained were validated via NTA, TEM and Western blotting (Figure 1). The NTA results revealed the diameter distribution of the EVs, which was consistent with the a priori knowledge of exosome-like small EVs, and TEM clearly revealed the shapes of the small EVs. Western blotting further confirmed the expression of three EV markers, TSG101, Alix and CD63, in ADEVs and NDEVs, and the expression of the astrocyte marker GFAP for ADEVs and the neuronal marker SNP25 for NDEVs. These results confirmed the successful acquisition of ADEVs and NDEVs.

## Group Differences in BDNF, TNF-α, SOD1 and MDA

Regarding neuroplasticity markers and neuroinflammatory markers in ADEVs, after adjusting for age and sex, the EP had a significantly lower concentration of BDNF than did the HC, whereas the concentration of TNF-α was significantly greater. However, there was no significant difference in the concentration of CD81 between the two groups, and the results remained robust after normalization for CD81 in the sensitivity analysis. See Table 2 and Figure 2 for details.

With respect to the concentrations of oxidative stress markers in NDEVs, after adjusting for sex and age, the concentration of SOD1 in NDEVs from EP was significantly lower than that in the HC, whereas the concentration of MDA and CD81 was significantly higher. The results remained robust after CD81 normalization in the sensitivity analysis. See Table 2 and Figure 3 for details.

In the subgroup analyses of patients with epilepsy, a total of 49 patients in EP were divided into focal epilepsy (27 [55.1%]) and generalized epilepsy (22 [44.9%]) subgroups; 11 (22.4%) were classified as having drug-refractory epilepsy, whereas 38 (77.6%) were classified as having drug-responsive epilepsy. There was no significant difference in CD81, SOD1, MDA, CD81-normalized SOD1 or CD81-normalized MDA levels in NDEVs, nor was there any difference in CD81, TNF-α, BDNF, CD81-normalized TNF-α, or CD81-normalized BDNF in ADEVs between the focal epilepsy group and the generalized epilepsy group. SOD1- and CD81-normalized SOD1 levels were significantly lower

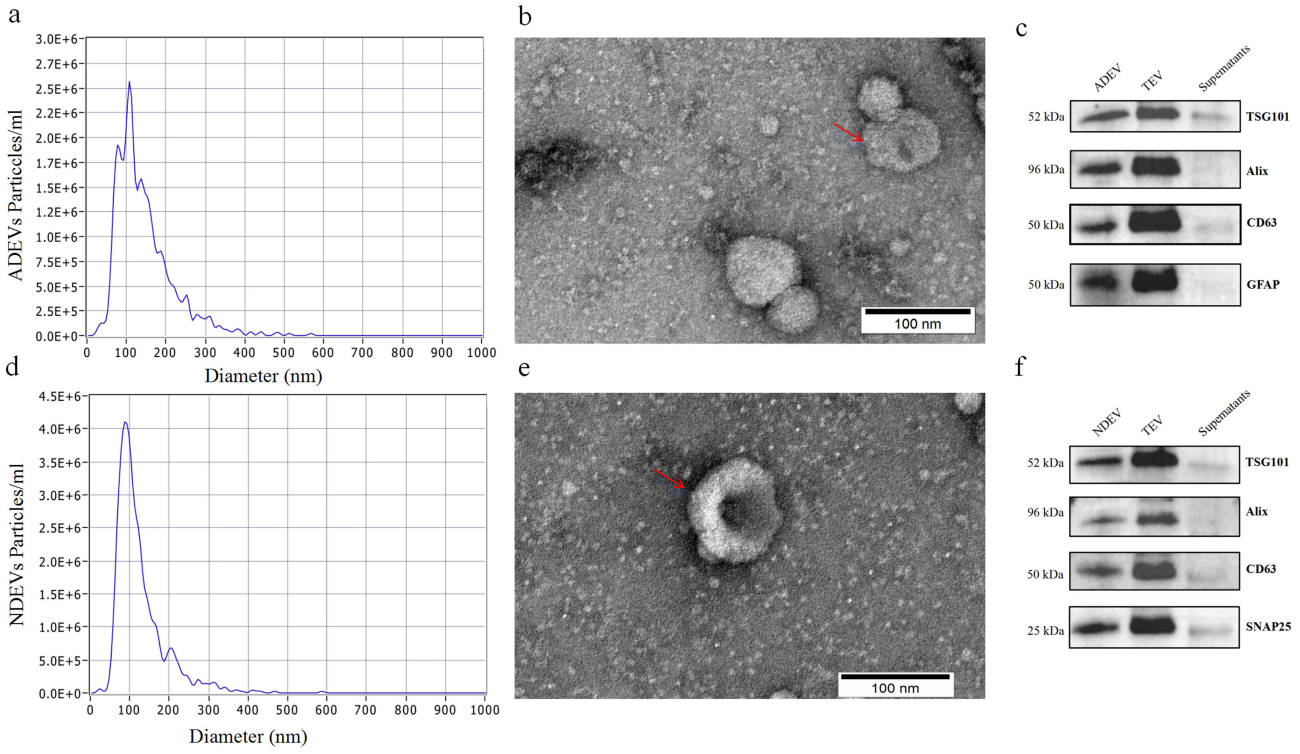


**Figure 1** Confirmation of ADEVs and NDEVs. (**a** and **d**) The results of nanoparticle tracking analysis show the diameter distribution of the EVs (ADEVs: diameter: 111.5 nm, Concentration: $5.2\times10^{9}$ particles/mL; NDEVs: diameter: 88.0nm, Concentration: $2.1\times10^{10}$ particles/mL); (**b** and **e**) The transmission electron microscopy image of ADEVs and NDEVs, the images clearly shows the exosomes-like shape (red arrows); (**c** and **f**) Western blotting results of ADEVs and NDEVs sample: three exosomes marker cluster of differentiation (CD) 63, CD81, tumor susceptibility (TSG) 101, an astrocyte marker glial fibrillary acidic protein (GFAP) for ADEVs and a neuron marker synaptosomal associated protein 25kDa (SNAP25) were identified.


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**Table 2** Comparisons of EMMs of the Target Protein Concentrations in ADEVs AND NDEVs Between EP and HC Group

| | EP EMM (95% CI) | HC EMM (95% CI) | Mean Difference EP-HC (95% CI) | *P* |
|---|---|---|---|---|
| In ADEVs | | | | |
| CD 81 (pg/mL) | 71.82(60.89, 82.74) | 61.59 (45.30, 77.92) | 10.22(−9.54, 29.89) | 0.303 |
| TNFα(pg/mL) | 104.40 (93.56, 115.23) | 53.02 (36.83, 69.23) | 51.36 (31.85, 70.82) | <0.001 |
| BDNF (pg/mL) | 349.27 (251.40, 447.14) | 803.8 (657.60, 950.11) | −454.58(−630.82, −278.34) | <0.001 |
| CD81 normalized TNFα(pg/mL) | 121.88 (104.97, 138.79) | 73.87 (48.59, 99.15) | 48.01 (17.55, 78.46) | 0.002 |
| CD81 normalized BDNF(pg/mL) | 446.35(301.76, 59.9) | 994.34 (778.27, 1210.42) | −547.98 (−808.35, −287.65) | <0.001 |
| In NDEVs | | | | |
| CD 81 (pg/mL) | 146.64 (124.45, 168.83) | 102.91 (69.75, 136.06) | 43.73(3.78, 836.68) | 0.032 |
| SOD1 (pg/mL) | 167.46(146.67, 188.25) | 208.79(177.73, 239.86) | −41.33 (−78.77, −3.90) | 0.031 |
| MDA (pg/mL) | 81.71(74.49, 88.92) | 27.26 (16.48, 38.03) | 54.45 (41.46, 67.44) | <0.001 |
| CD81 normalized SOD1(pg/mL) | 192.02 (129.03, 254.99) | 457.92 (363.81, 552.04) | −265.91 (−379.31, −152.05) | <0.001 |
| CD81 normalized MDA (pg/mL) | 94.61(76.59, 112.73) | 63.99 (36.99, 90.98) | 30.67 (−1.85, 63.20) | 0.064 |

**Notes**: Age and sex are added as covariates.

**Abbreviations**: EP, epilesy; HC, health control; EMM, estimated marginal mean; CI, confidence interval; CD, cluster of differentiation; ADEVs, astrocyte-derived extracellular vesicles; NDEVs, neuron-derived extracellular vesicles; TNF, tumour necrosis factor; BDNF, brain-derived neurotrophic factor; SOD, superoxide dismutase; MDA, malondialdehyde.

in the drug-refractory epilepsy group than in the drug-responsive epilepsy group, whereas CD81-normalized TNF-α levels were significantly greater; the levels of the other target proteins were not significantly different between these two groups (Table S1).

## Correlations Between Neuroplasticity and Neuroinflammation and Oxidative Stress

In patients with epilepsy, Spearman correlation analysis revealed that BDNF levels in ADEVs were negatively correlated with TNF-α levels (ρ=−0.497, P<0.001) in ADEVs and MDA levels (ρ=−0.535, P<0.001) in NDEVs and positively correlated with SOD1 levels (ρ=−0.456, *P*=0.001) in NDEVs. In addition, TNF-α levels in ADEVs were negatively correlated with SOD1 levels in NDEVs (ρ=−0.292, *P*=0.04) and positively correlated with MDA levels in NDEVs (ρ=0.389, *P*=0.006), whereas SOD1 levels in NDEVs were negatively correlated with MDA levels (ρ=−0.537, P<0.001) (Figure 4). After CD81 normalization, the above correlation results remained robust (*P*<0.05) (Figure 5), and the correlation heatmap shows detailed results (Figure S1).

# Discussion

The present study innovatively used ADEVs and NDEVs as brain-derived biomarkers to explore markers of neuroplasticity, neuroinflammation and oxidative stress and found in vivo evidence of impaired neuroplasticity in patients with epilepsy compared with HC. Furthermore, we found that these alterations may be associated with CNS increased neuroinflammation and oxidative stress.

## Astrocytes, Neuroplasticity, and Epilepsy

Astrocytes, the most abundant glial cell type in the CNS and one of the major sources of BDNF in the CNS, influence neuroplasticity by releasing BDNF to regulate neuronal survival, growth, differentiation, and synaptic plasticity.[5] BDNF activates downstream signaling pathways, such as the PI3K–Akt and MAPK–Erk pathways, which are involved in the regulation of neuronal structural and functional plasticity, by binding to specific receptors (eg, TrkB receptors) on neurons.[34] For example, in the hippocampus, the release of BDNF promotes the formation and maturation of synapses and enhances the efficiency of synaptic transmission, thereby contributing to learning and memory processes.[35] Patients with epilepsy, including focal epilepsy,[36] temporal lobe epilepsy,[37] and unclassified epilepsy subtypes,[38,39] exhibited significantly reduced serum brain-derived BDNF concentrations. Sun et al demonstrated that this reduction is correlated with epilepsy severity, psychiatric comorbidities, and cognitive impairment.[40] However, other studies have found no differences or have reached opposite conclusions.[41–43] The theory of the dual mechanism of action of BDNF is one of the

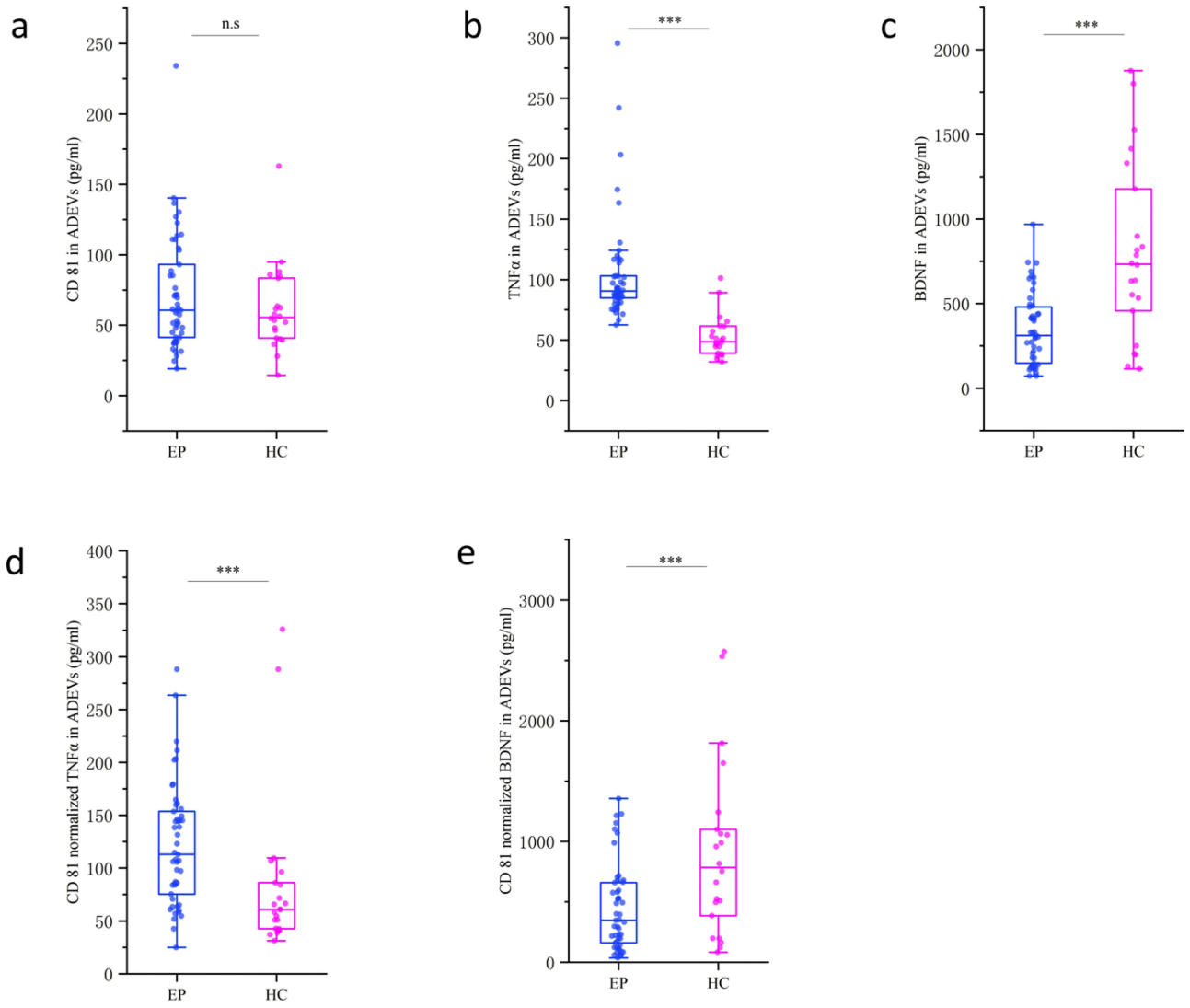


**Figure 2** Boxplots of target protein concentrations in ADEVs between EP and HC group. (**a**) CD81 in ADEVs; (**b**) TNFα in ADEVs; (**c**) BDNF in ADEVs; (**d**) CD81 normalized TNFα in ADEVs; (**e**) CD81 normalized BDNF in ADEVs.
**Notes**: n.s.: not significant; ***$P < 0.001$.

explanations for this paradox, in which the reduction in BDNF during epileptogenesis may be associated with certain aspects of the pathogenesis of epilepsy. For example, cell-mediated immune activation and inflammatory processes cause dysregulation of BDNF, leading to a reduction in hippocampal neurogenesis and neurodegeneration, which affects epileptogenesis, and the reduction in BDNF levels in this case is a manifestation of disease progression.[44] Conversely, BDNF may have neuroprotective effects. For example, the BDNF–TrkB signaling pathway affects synaptic plasticity in the hippocampal neurons of epileptic rats, which may protect neurons during seizures by maintaining or repairing synaptic plasticity and attenuating the damage caused by seizures.[45] Notably, the above studies focused on changes in peripheral blood BDNF, but epilepsy is a brain disease. Thus, we explored BDNF levels in astrocytes directly through ADEVs, a brain-derived biomarker, and found that BDNF was significantly decreased in the EP of ADEVs compared with HC, providing in vivo evidence that is more reflective of the brain condition. Thus, our findings lend more support to the theory that decreased BDNF is a consequence of repetitive seizures, which may lead to impaired neuroplasticity in epilepsy.


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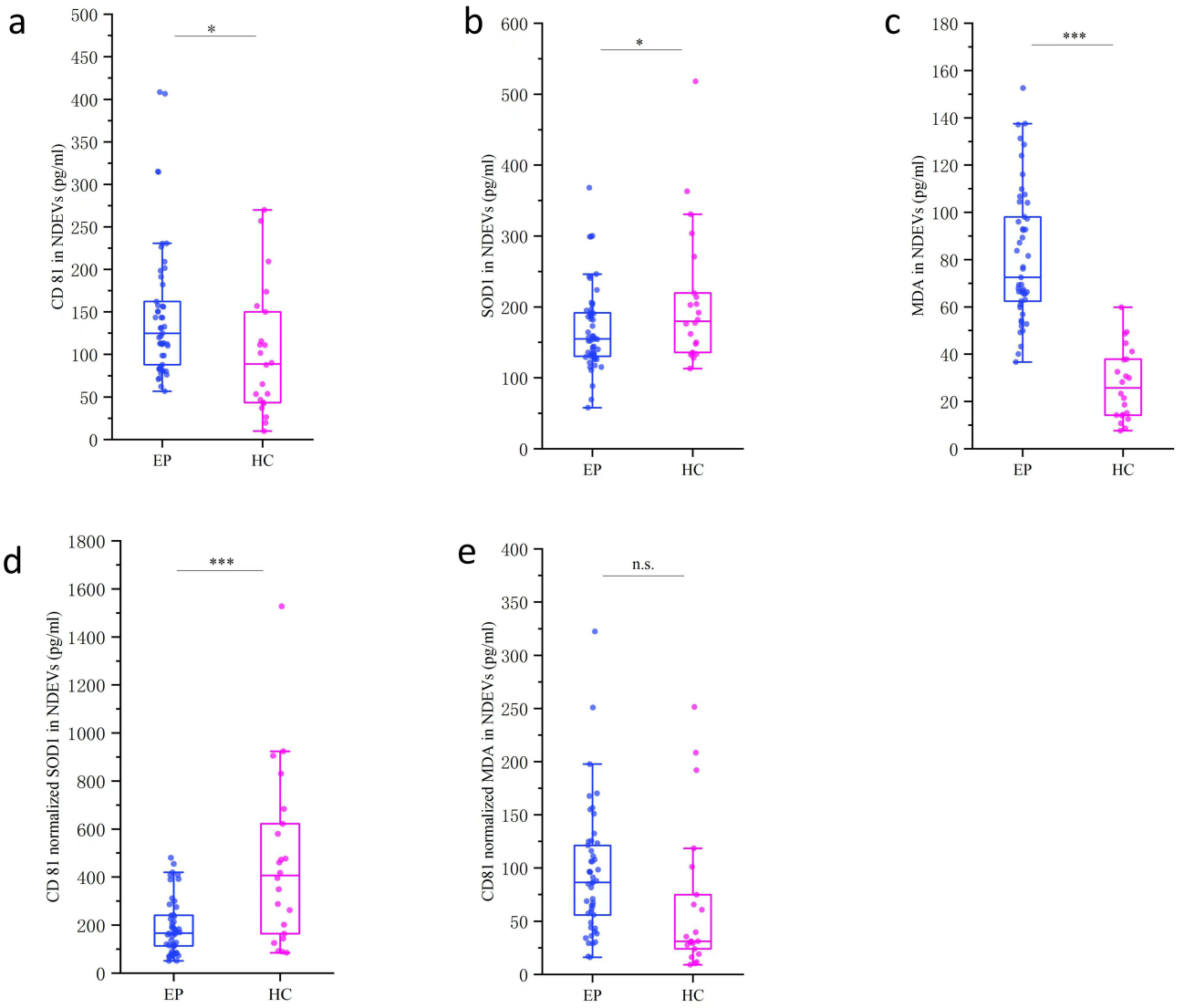


**Figure 3** Boxplots of target protein concentrations in NDEVs between EP and HC group. (**a**) CD81 in NDEVs; (**b**) SOD1 in NDEVs; (**c**) MDA in NDEVs; (**d**) CD81 normalized SOD1 in NDEVs; (**e**) CD81 normalized MDA in NDEVs.
**Notes**: n.s.: not significant; *$P < 0.05$; ***$P < 0.001$.

## Neuroinflammation, Oxidative Stress and Epilepsy

The role of neuroinflammation and oxidative stress in the pathophysiology of epilepsy has received much attention in recent years. Evidence suggests that significant crosstalk exists between neuroinflammation and redox-based signaling cascades.[15] Oxidative posttranslational modifications have been shown to directly affect the function of major neuroinflammatory mediators.[15] As transcriptional regulators such as NF-κB are activated by reactive oxygen species, neuroinflammation is controlled at the transcriptional level, which in turn increases CNS inflammation levels.[46,47] In addition, neuroinflammation induces an increase in the expression and activity of NADPH oxidative enzymes, which in turn decreases SOD1 and catalase activity and increases reactive oxygen species and MDA levels, leading to excessive oxidative stress.[15] Research on in vivo markers of oxidative stress in epilepsy has focused on peripheral blood and revealed a decrease in the activity of epileptic peripheral blood antioxidants such as SOD1, GSH and vitamin C and an increase in the levels of lipid peroxides such as MDA in patients with epilepsy.[12,48,49] The present study provides in vivo evidence of increased oxidative stress in the neurons of patients with epilepsy by revealing decreased levels of SOD1 and increased MDA through the brain-derived biomarkers of NDEVs.

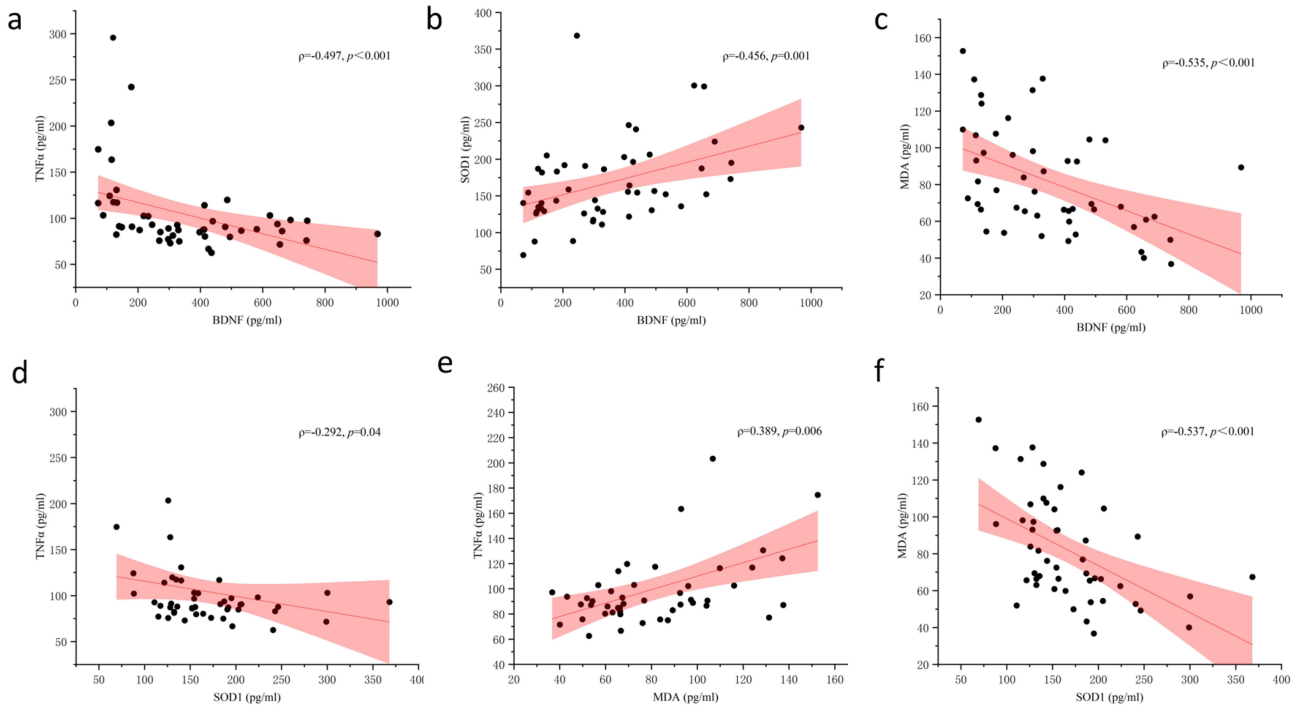


**Figure 4** Spearman rank correlation scatter plot between BDNF and markers of neuroinflammation and oxidative stress. (**a**) BDNF and TNFα; (**b**) BDNF and SOD1; (**c**) BDNF and MDA; (**d**) SOD1 and TNFα; (**e**) MDA and TNFα; (**f**) SOD1 and MDA.

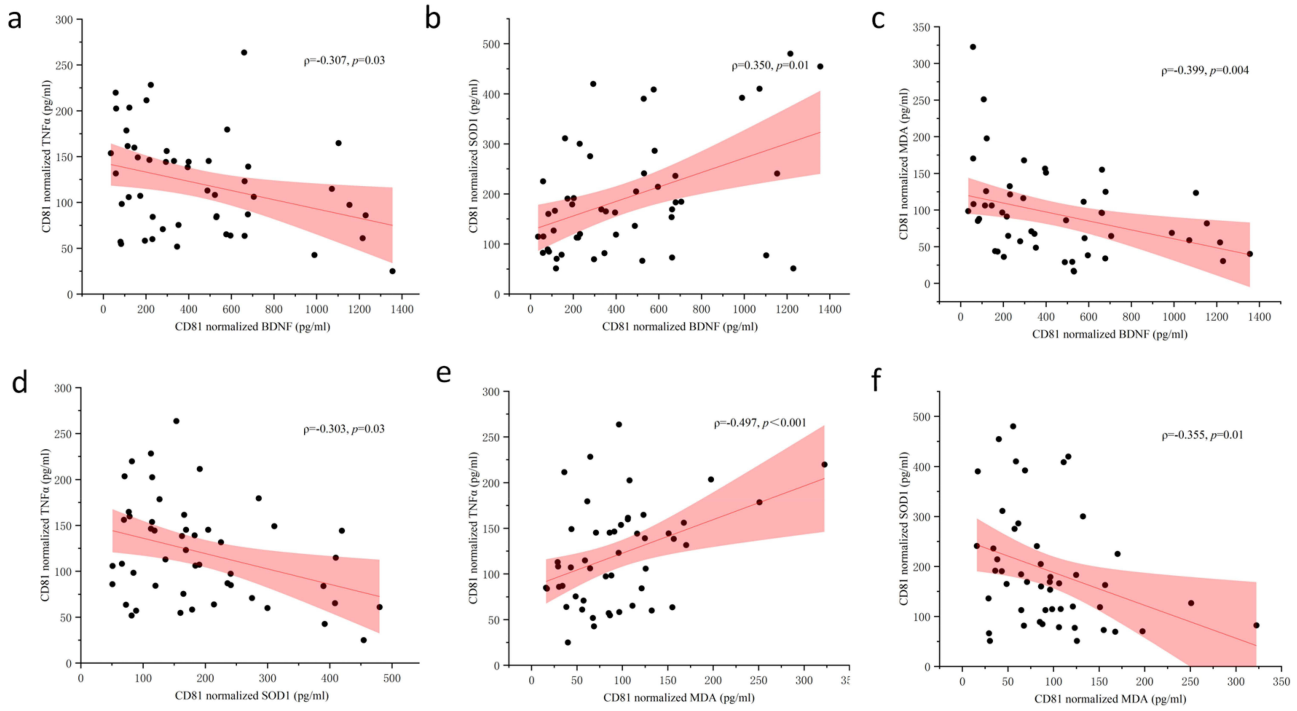


**Figure 5** Spearman rank correlation scatter plot between CD81 normalized BDNF and neuroinflammatory and oxidative stress markers (after CD81 normalisation). (**a**) CD81 normalized BDNF and CD81 normalized TNFα; (**b**) CD81 normalized BDNF and CD81 normalized SOD1; (**c**) CD81 normalized BDNF and CD81 normalized MDA; (**d**) CD81 normalized SOD1 and CD81 normalized TNFα; (**e**) CD81 normalized MDA and CD81 normalized TNFα; (**f**) CD81 normalized SOD1 and CD81 normalized MDA.


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With respect to epileptic neuroinflammatory markers, studies have revealed increased levels of inflammatory factors such as interleukin-6 (IL-6), interleukin-1-beta (IL-1β) and TNFα in the cerebrospinal fluid of patients with epilepsy.[50,51] Because of the importance of astrocytes in the CNS inflammatory response, the present study revealed increased TNFα levels in ADEVs, which is consistent with the findings of those studies, providing further in vivo evidence of central increased neuroinflammation in patients with epilepsy. In exploratory studies, SOD1 levels were found to be significantly lower in NDEVs in the drug-refractory epilepsy group than in those in the drug-responsive epilepsy group, and CD81-normalized TNF-α levels in ADEVs were found to be significantly higher than those in the drug-responsive epilepsy group, which is in agreement with the findings of previous studies,[12,50,52] suggesting the potential value of oxidative stress and neuroinflammation markers in predicting drug-refractory epilepsy.

## Correlations Between Neuroplasticity, Neuroinflammation and Oxidative Stress in Epilepsy

Several cellular and molecular mechanisms are activated in epileptic neuroinflammatory states. Activation of microglia and astrocytes leads to a chronic inflammatory process in epilepsy, and the inflammatory response induces a number of changes in astrocytes, including effects on BDNF synthesis.[53] Moreover, intracellular signaling pathways activated by BDNF binding to TrkB receptors on neurons are also disturbed in the epileptic neuroinflammatory environment, leading to abnormalities in cellular processes related to neuroplasticity, such as axon growth and altered dendritic spine density.[54] At the animal level, activation of the neuroinflammatory response is followed by inhibition of long-term potentiation (LTP) in the mouse hippocampus, accompanied by a decrease in BDNF levels.[55] These results suggest that epileptic neuroinflammation affects synaptic connectivity and functional plasticity between neurons by influencing the synthesis and secretion of BDNF in astrocytes. Relatively few clinical studies have been conducted; one study analyzed brain tissue samples from patients with epilepsy and revealed elevated levels of neuroinflammation and abnormal BDNF expression in epileptic focal areas. In addition, these patients tended to exhibit cognitive dysfunction, which is strongly associated with neuroplasticity.[56] These findings suggest that in patients with epilepsy, neuroinflammation may impair neuroplasticity by affecting BDNF. Using a ADEVs as convenient peripheral blood brain-derived biomarkers, the present study revealed a negative correlation between BDNF and TNFα in ADEVs, providing additional in vivo evidence for the influence of increased neuroinflammation on epileptic neuroplasticity.

Oxidative stress interferes with the normal physiological function of astrocytes, which may in turn affect BDNF synthesis.[57] In a mouse model of epilepsy, the level of oxidative stress in the brain was significantly increased after seizures, whereas the synthesis of BDNF in astrocytes was altered. With repeated seizures, the cellular mechanisms associated with BDNF synthesis are disrupted under sustained damage caused by oxidative stress, resulting in decreased synthesis. Additionally, altered neuroplasticity, such as reduced synaptic transmission efficiency in the hippocampus and difficulty in LTP induction, was observed.[58] From a clinical research perspective, patients with epilepsy treated with certain antiepileptic drugs with antioxidative stress effects, such as sodium valproate,[10] exhibited increased serum BDNF levels and cognitive function recovery. These results suggest that epileptic oxidative stress affects neuroplasticity by affecting the BDNF-neuroplasticity pathway. In the present study, for the first time, we used peripheral blood ADEVs and NDEVs as brain-derived biomarkers tools and reported that decreased BDNF levels in the ADEVs of patients with epilepsy were positively correlated with SOD1 levels in NDEVs and negatively correlated with MDA levels, suggesting that impaired neuroplasticity in patients with epilepsy may be related to increased oxidative stress.

# Limitations

There were several limitations in the present study. First, the study was conducted with children, which may lead to potential heterogeneity originating from a wider age range, thus limiting the applicability of the results to other age groups. Second, microglia are the most abundant resident immune cell population in the CNS and play the most critical role in neuroinflammation, However, further research is limited due to the lack of highly specific surface markers.Third, although BDEVs can function as brain-derived biomarkers, they have the nonnegligible drawback of low spatial resolution, and subsequent methodological innovations are needed to solve this problem. Fourth, owing to research

funding considerations, we were unable to test additional alternative markers of neuroplasticity, neuroinflammation and oxidative stress. A comprehensive assessment of known relevant markers would provide more convincing conclusions. Furthermore, the present study is essentially a case–control study and does not establish a causal relationship among neuroplasticity, neuroinflammation, oxidative stress and the epilepsy disorder itself in patients with epilepsy. In particular, we provide in vivo evidence confirming that there may be altered neuroplasticity in epilepsy and that it may be associated with increased neuroinflammation and oxidative stress.

## Conclusion

The use of ADEVs and NDEVs as brain-derived biomarkers in this study provides in vivo evidence that epilepsy may have impaired neuroplasticity and may be associated with increased neuroinflammation and oxidative stress. The novel findings of our study may provide new insight into the pathogenic mechanisms of epilepsy and may contribute to the development of more effective biomarkers for epilepsy. Subsequent studies should involve larger cohorts and multi-omics approaches to discover and validate epilepsy-specific biomarkers, facilitating improved early diagnosis, disease monitoring, and prognosis assessment.

## Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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