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RESEARCH ARTICLE

Effect of *APOE* ε4 allele on levels of apolipoproteins E, J, and D, and redox signature in circulating extracellular vesicles from cognitively impaired with no dementia participants converted to Alzheimer's disease

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Abstract

Introduction: The substantial link between apolipoprotein E (*APOE*) ε4 allele and oxidative stress may underlie enhanced Alzheimer's disease (AD) risk. Here, we studied the impact of *APOE* ε4 on the level of apolipoproteins with antioxidant activities along with oxidative markers in circulating extracellular vesicles (cEVs) and plasma from cognitively impaired-not demented (CIND) individuals converted to AD (CIND-AD).

Methods: Apolipoproteins E, J, and D and antioxidant response markers were determined in cEVs and plasma using immunoblotting, electrochemical examination, and spectrofluorimetry.

Results: Total antioxidant capacity and apolipoprotein D levels in cEVs, as judged by regression analysis and cognitive performance correlations, allowed us to differentiate CIND *APOE* ε4 carriers from controls and to predict their progression to AD 5 years later.

Discussion: Our findings support the pathological redox linkage between *APOE* ε4 and AD onset and suggest the use of cEVs oxidative signature in early AD diagnosis.

KEYWORDS
Alzheimer's disease, apolipoprotein E, extracellular vesicles, oxidative stress

1 | BACKGROUND

Alzheimer's disease (AD) is an age-related brain disorder and the leading cause of dementia worldwide. People with AD display an altered cognitive performance associated with gradual memory loss.[1] Despite remarkable breakthroughs in the understanding of the disease and globalized research efforts, no early evidence-based diagnosis or effective treatment therapies are yet available.[2]

Apolipoprotein E (*APOE*) ε4 allele is the most important predictor factor, besides age, of subsequent progression to AD.[3] Remarkable ethnicity-specific effects of *APOE* ε4 on AD risk were reported and it is suggested that *APOE* ε4-related cognitive impairment is racial-dependent.[4,5] The apoE protein, mainly produced by liver parenchymal cells, is fundamental to maintain lipoproteins' structural integrity and to promote their solubilization in blood circulation.[6] However, other tissues and organs synthesize significant amounts of apoE, most

prominently the brain where apoE is essential to modulate cerebral homeostasis by managing lipid transport and the efflux of key moieties through the blood-brain barrier (BBB).[7] Among the nine apolipoproteins synthetized in the central nervous system (CNS), apoE, apoJ, and apoD are the most abundant and there is evidence that some of the key neuroprotective processes including the clearance of neurotoxic amyloid beta peptide and the management of oxidative stress are regulated in a cooperative manner among these apolipoproteins.[8,9] Although the relationships among apoE, J, and D proteins and brain function is well documented, the precise pathological implication of *APOE* ε4 in apolipoprotein regulation and AD risk remains elusive.

The complex pathophysiology of AD prevents the establishment of well-defined and clear causes of the disease. Nonetheless, there is a broad recognition of the role of oxidative stress in AD etiopathogenesis because it is associated with cellular and molecular abnormalities observed in AD such as senile plaque formation,[10] hyperphosphorylation of tau protein,[11] decreased synaptic plasticity,[12] neuroinflammation,[13] and loss of mitochondrial function.[14]

Mounting evidence from studies in cell lines, transgenic animals, and humans now relate *APOE* ε4 to higher oxidative insults in AD. The degree of oxidative brain damage was strongly associated with the ε4 allele rather than the ε3 and ε2 isoforms in AD patients.[6] Our previous work showed increased lipid peroxidation associated with lower glutathione levels and reduced activities of glutathione peroxidase and catalase in the hippocampus and frontal cortex of *APOE* ε4 AD patients compared to *APOE* ε3 patients, further suggesting a higher impact of the *APOE* ε4 variant on redox-mediated brain damage.[15,16] Moreover, data from *APOE* knock-out and human *APOE* targeted replacement mice studies suggest that *APOE* ε4–mediated neurotoxicity is an early event in AD pathology.[17,18]

Interestingly, several studies showed that many blood-based oxidative stress biomarkers are consistent with oxidative brain changes and AD risk.[19,20] Given that oxidative stress is one of the earliest pathological manifestations in AD and that brain oxidative damage could be extended to the blood compartment, the analysis of circulating oxidative stress markers among pre-AD *APOE* ε4 carriers could be a useful strategy in early AD diagnosis and monitoring.

Extracellular vesicles (EVs) form a heterogeneous group of nanoparticles that differ in size, biogenesis process, cellular origin, and biophysical properties.[21] Exosomes (50 to 150 nm) and microvesicles (50 to 500 nm) are released from healthy or experimentally induced cells, whereas apoptotic bodies (up to 1 μM) are generated after cell death.[21] EVs produced from normal cell processing are loaded with a variety of molecular constituents that are believed to maintain vital processes such as cell-to-cell communication.[22] Also, it is suggested that EVs could cross the BBB from the bloodstream to the CNS and vice versa,[23,24] which implies that circulating EVs (cEVs) may cause and/or reflect a possible alteration in the CNS. In this regard, cumulative clinical evidence supports the reliability of blood EV-based biomarkers in AD diagnosis.[22,25,26] Our previous works have shown that cEVs' protein cargo provided a fair classification between mild cognitive impairment (MCI) and AD patients,[27] predicted the conversion of cognitively impaired-not demented (CIND) patients to AD,[28] and was able to differentiate early and moderate AD stages.[29] Moreover, we have demonstrated that some neurotrophic and inflammatory factors measured in cell neuronal-derived EVs (nEVs) are regulated differently under stress treatment.[30]

To provide a comprehensive insight into the potential interaction between *APOE* ε4 variant and oxidative stress state in early AD development, our study aims to (1) determine a profile of redox factors measured in plasma and in cEVs that can predict the progression of participants in preclinical stages of dementia to AD, and (2) evaluate the impact of the presence of *APOE* ε4 on stress regulation and AD risk.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed the literature using PubMed. Publications describing the role of oxidative stress in functional and structural brain changes observed in Alzheimer's disease (AD) with regard to the pathological implication of apolipoprotein E (*APOE*) ε4 variant are cited throughout the article.
2. **Interpretation**: Our findings show that *APOE* ε4 status increased the predictive value for AD conversion among cognitively impaired-not demented (CIND) participants through poor management of multiple oxidative systems and underlie the utility of peripheral extracellular vesicles (cEVs) in early AD diagnosis.
3. **Future directions**: This article provides further understanding of the influence of *APOE* genotype in modulating cEVs' content and draws attention on the systemic and brain redox failure related to *APOE* ε4 status. Upon replication of results in larger sample size cohorts, future investigations should consider the association of *APOE* genotyping in EV-associated biomarker AD studies.

2 | METHODS

2.1 | Participants and data source

Data were taken from the Canadian Study of Health and Aging (CSHA), a multiphase, longitudinal, population-based cohort study of the epidemiology of dementia in Canada. Women and men aged 65 years and over were randomly selected from urban and surrounding rural areas in all Canadian provinces. Information on risk factors was collected using a self-administered questionnaire (see Table S1 in supporting information). Participants were clinically evaluated in CSHA-1 (1991–1992) by a nurse, a physician, and a neuropsychologist, and a consensus diagnosis was reached between the physician and the neuropsychologist according to the published criteria of Diagnostic and Statistical Manual of Mental Disorders, 3rd Edition, Revised (DSM-III-R) for dementia, the National Institute of Neurological and Communicative Disorders

and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) for AD, and the International Classification of Disease, 10th Edition (ICD-10) for vascular dementia (VaD).[28]

Updated diagnostic criteria were used in CSHA-2 (1996–1997) and CSHA-3 (2001–2002) for dementia and AD (DSM-IV) and VaD (National Institute of Neurological Disorders and Stroke–Association Internationale pour la Recherche et l'Enseignement en Neurosciences).[28] Participants with cognitive impairment, but who failed to meet the standardized criteria for dementia, were classified as CIND. In CSHA, CIND was a clinical entity referring to a potentially reversible condition between normal cognition and dementia, which accordingly, differs from the MCI state and tends to describe the pre-dementia stages and the preclinical aspects of the disease.[28,31]

In the present nested case-control study, we selected from the CSHA-2 blood bank 15 participants clinically diagnosed with CIND who converted 5 years later in CSHA-3 to AD-dementia (CIND-AD). These CIND-AD cases were age-matched with 21 controls clinically evaluated with no cognitive impairment at CSHA-2 and who remained as such in CSHA-3, with respect to inclusion and exclusion criteria (see Table S1). All participants were genotyped for the presence of *APOE* ε4 allele based on the method of Zivelin et al. (see Table S2 in supporting information).[32] Plasma samples collected at CSHA-2 were used for cEVs isolation and all parameters analysis. An informed written consent was filled out by participants prior to enrollment to the study. All procedures were approved by the INRS-IAFSB ethics committee (CER19-532).

2.2 | Isolation and characterization of extracellular vesicles

The isolation of cEVs was assessed as described in our previous works,[27,29,30] and characterized according to the last methodological guidelines to study EVs.[33]

2.2.1 | Nanoparticles tracking analysis

The NanoSight NS300 system (Malvern Panalytical Inc.) was used to determine the concentration and size distribution of cEV preparations. Isolated non-lysed cEVs were diluted (1/1000) in filtred milli-Q® water before injection. All measurements were conducted under uniform and synchronized conditions. The video images were analyzed using the NanoSight NTA 3.2 analytical software.

2.2.2 | Transmission electron microscope

The morphology of isolated cEVs was analyzed using the Hitachi H-7100 transmission electron microscope. To ensure minimal spectral changes, samples were fixed with 2% paraformaldehyde for 5 minutes and then placed on a copper grid with carbon-coated formvar film. The grid was incubated with 2% uranyl acetate (w/v) and excess solution was removed by blotting. Finally, cEVs were observed using a 40000X magnification at 75 kV.

2.2.3 | Western blot analysis of EVs markers

To analyze vesicular cargo, isolated cEVs were lysed with RIPA buffer (5 mM EDTA, 50 mM Tris buffer, 0.1% sodium dodecyl sulfate, 150 mM sodium chloride, 1% sodium deoxycholate, and 1% Igepal) mixed with phosphatase and protease inhibitors (1:100% v/v). cEV lysate proteins were then transferred into polyvinylidine difluoride (PVDF) membranes and incubated with the following label primary antibodies: mouse anti-calnexin (1/500; Sc-23954, Santa Cruz Biotechnology, SCBT), mouse anti-tetraspanin CD63 (1/500; Sc-5275, SCBT), mouse anti-Iba1 (1/500; sc-32725, SCBT), mouse anti-GFAP (1/1000; HPA056030, Sigma), and rabbit anti-TSG101 (1/2000; MBS7605273, MyBiosource). Membranes were then washed and incubated with respective immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked secondary antibodies: anti-mouse (1/1000; 7076S, Cell Signaling Technology, SCT) and anti-rabbit (1/2000; 7074S, SCT). The enhanced chemiluminescence (ECL) substrate kit (Bio-Rad) was used for immunoblot detection and signal analysis was carried out using the ChemiDoc imaging system.

2.3 | Plasma total antioxidant capacity

Plasma total antioxidant capacity (TAC) was measured by the free radical analyzer Apollo 4000 (World Precision Instruments). The hydrogen peroxide (H_2O_2) sensor was used to estimate the real-time and direct quantitative measurement of degraded H_2O_2 by plasma samples. A calibration curve was generated according to a linear response of the H_2O_2 electrode upon the addition of different amounts of H_2O_2 (H1009, Millipore). Sample analysis was preceded by adding 255 μg of plasma proteins to 2 mL of PBS (1X, pH 7.4) followed by the injection of 2 μmol of H_2O_2 once the sensor was stabilized. The picoampere variations in the reaction mixture were measured to assess the plasma's ability to reduce H_2O_2.

2.4 | Proteasome activity assay

Plasma 20S proteasome lytic activity was evaluated using the fluorogenic Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) substrate that targets the chymotrypsin-like activity of the proteasome.[19] Briefly, 10 μl of each plasma sample was incubated at room temperature for 15 minutes with 10% sodium dodecyl sulfate (SDS). Nonactivated and activated plasma samples (10 μl) were placed with 30 μl of assay buffer and 10 μl of the fluorogenic peptide at a final concentration of 100 μM with and without the irreversible inhibitor Marizomib (250 nM). Finally, samples were incubated at 37°C for 30 minutes and the released fluorescent AMC was measured at

350/440 nm using a Hitachi F-2000 spectrofluorometer. Results were expressed in AMC/min/μl of plasma.

2.5 | Immunoblotting analysis of protein carbonyls and apolipoproteins

Plasma and cEV levels of protein carbonyls, apoE, apoJ, and apoD were measured by immunoblotting. Briefly, 20 μg of plasma and cEV proteins were loaded on 10% polyacrylamide gels and separated by electrophoresis. Gels were electroblotted onto PVDF membranes that were incubated at 4°C overnight with the following primary antibodies: clusterin polyclonal rabbit antibody (1/2500; MyBiosource), monoclonal 2B9 mouse anti-apoD (1/5000),[34] and anti-rabbit apoE (1/500; sc-390925, SCBT). Concerning protein carbonyls, blotted PVDF membranes were incubated with 1 mM of di-nitrophenol hydrazine (DNPH) derivative for 1 hour and then blocked with 5% skim milk overnight at 4°C before the addition of the primary anti-mouse DNP antibody (1/2000; D9656, Sigma). After removing excess primary antibodies, membranes were incubated with HRP-conjugated secondary IgG: anti-rabbit (1:2000; 7074S, SCT) or anti-mouse (1/1000; 7076S, SCT). The immunoreactive signals were visualized by adding the ECL reagent (Bio-Rad) and their intensities were quantified by densitometric scanning of blots using the FluorChem luminescent system. Total proteins were stained with Coomassie blue as controls to normalize the loading.

2.6 | Statistical analysis

The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess normal distribution. Statistical analysis of the clinical, biochemical, and specific study parameters was performed using the Student's *t* test and the one-way analysis of variance (ANOVA) on the SPSS v18.0 software. The nonparametric Mann-Whitney and Kruskal-Wallis tests followed by Dunn test were applied for data that are not normally distributed. A statistical correlation was performed by a linear regression analysis using the Pearson correlation coefficient. Areas under the receiver operating characteristic (ROC) curves (AUC) were calculated using GraphPad Prism v7.0 software. The Mini-Mental State Examination (MMSE) scores derived from the Modified Mini-Mental State (3MS) examination were used to evaluate the global cognitive function between the study participants.

Data from chemiluminescence analysis are expressed as arbitrary units. An arbitrary unit represents the ratio between the marker band intensity and the corresponding total protein intensity stained with the Coomassie blue. Results are considered significant at $P < .05$.

3 | RESULTS

3.1 | Baseline and specific cohort characteristics

Demographic, clinical, and biochemical data of the study population are displayed in Table 1. The sex ratio and age were well balanced between the two groups. The levels of lipid and inflammatory parameters, as well as minerals and vitamins, were similar between cases and controls. Meanwhile, MMSE scores and body mass index (BMI) were significantly lower among CIND-AD cases compared to controls.

TABLE 1 Clinical and biochemical characteristics of the study participants

	Controls (n = 21)	CIND-AD (n = 15)
Clinical profile		
Age (y)	79.0 ± 5.2	81.4 ± 4.5
Sex ratio (M/F)	10/11	6/9
BMI (Kg/m^2)	26.5 ± 4.3	22.7 ± 4.4*
Education (y)	10.9 ± 3.7	8.8 ± 2.5
Score MMSE	27.7 ± 1.5	23.0 ± 1.9*
Genotype (*APOE* $\varepsilon4^-$/*APOE* $\varepsilon4^+$)	21/0	5/10
Lipid profile		
Total cholesterol (mmol/L)	4.4 ± 0.9	4.2 ± 0.8
Triglycerides (mmol/L)	1.5 ± 0.6	1.3 ± 0.5
oxLDL (U/L)	41.7 ± 11.4	38.8 ± 10.9
Inflammation profile		
TNF-α (pg/mL)	2.0 ± 0.9	2.3 ± 1.3
IL-6 (pg/mL)	1.8 ± 0.8	1.7 ± 1.2
CRP (mg/L)	3.2 ± 4.3	3.1 ± 3.4
Minerals & vitamin		
Cu (μmol/L)	13.0 ± 1.9	12.9 ± 1.5
Zn (μmol/L)	319 ±114.9	333.3 ± 91.8
Pb (μmol/L)	0.14 ± 0.6	0.22 ± 0.22
Vitamin D (nmol/L)	39.6 ± 22.7	35.9 ± 17.8

Note: Values are given as mean ± standard error mean.
Abbreviations: (−), $\varepsilon4$ non-carriers; (+), $\varepsilon4$ carriers; AD, Alzheimer's disease; *APOE*, apolipoprotein E; BMI, body mass index; CIND, cognitively impaired, no dementia; CRP, C-reactive protein; Cu, copper; F, female; IL-6, interleukin 6; M, male; MMSE, Mini-Mental State Examination; oxLDL, oxidized low-density lipoprotein; Pb, lead; TNF-α, tumor necrosis factor-alpha; y, years; Zn, zinc.
Student *t* test was used for statistical analysis with *, $P < .05$ compared to controls.

3.2 | Characterization of isolated cEVs

The collected transmission electron microscopy (TEM) images revealed the presence of cup-shaped structures with high contrast, which reflects the presence of a lipid-rich layer compatible with cEV morphological features (Figure 1A). NTA analysis showed similar curves distribution of cEV concentration between control and CIND-AD cases (Figure 1B). The cEVs associated markers (TSG-101 and CD63), as well as astrocytic glial proteins (Iba1 and GFAP), were detected by immunoblotting (Figure 1C and D). Calnexin was only found in SK-N-SH neuroblastoma lysate, which validates isolated cEV purity. The total protein amount in cEVs (3.24 ± 0.11 μg/μl) is 22-fold lower than in plasma (72.16 ± 0.73 μg/μl) and it is not different between the two

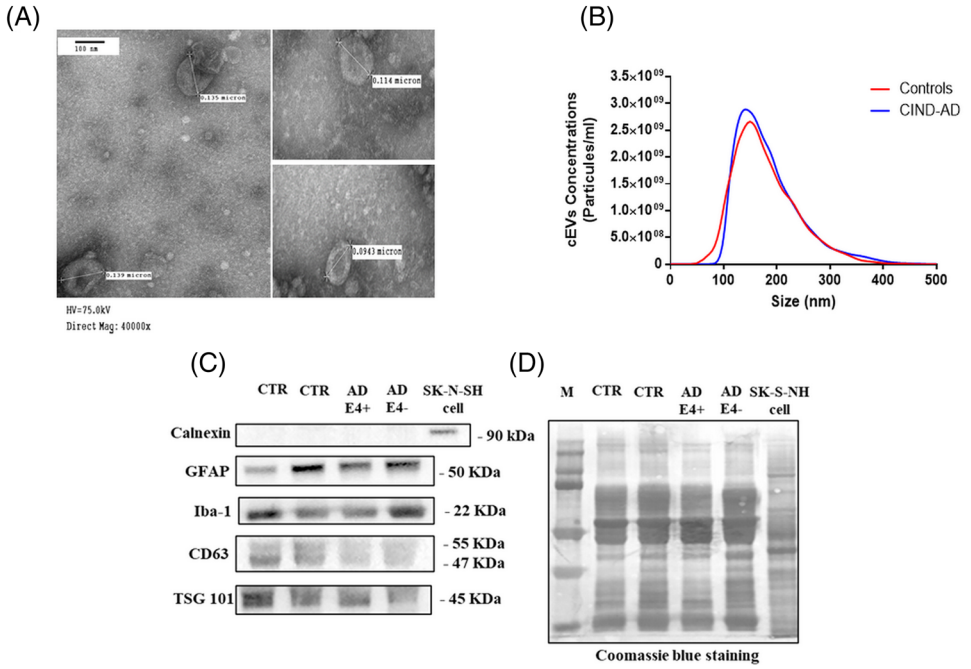

FIGURE 1 Visualization and characterization of circulating extracellular vesicles (cEVs) isolated from plasma. A, cEV images as acquired by transmission electron microscopy, bar represents 100 nm. B, Size distribution and concentration of cEVs examined by NTA using the NanoSight-NS300. C, Immunoblot detection of EV protein markers and non-associated proteins. D, Total cEV protein profile revealed by Coomassie blue staining

studied groups. This finding suggests the use of total protein load to normalize the data.

3.3 | Plasma and cEV levels of oxidative markers

A significant decrease in TAC was observed for the CIND-AD group (Figure 2A). Plasma and cEV levels of apoD were higher in CIND-AD participants compared to controls (Figure 2C and D). Interestingly, protein carbonyls content and apoJ/D ratio were statistically different in cEVs (Figure 2B and E) but not in plasma from CIND-AD (see Figure S1F-G in supporting information). Meanwhile, circulating-proteasome activity, apoJ, and apoE levels measured in both compartments were similar between the studied groups (see Figure S1A-E). Because BMI and sex could interfere with the regulation of the studied factors as confounders, the possible correlation was tested and found to be inconclusive (see Table S3 in supporting information). Our data indicate that TAC, cEV protein carbonyls, and cEV apoJ/D levels were correlated with the MMSE scores (Figure 3A, B, and E). Meanwhile, plasma and cEVs apoD were not associated with cognitive performance (Figure 3C and D).

3.4 | *APOE* ε4-driven effects on oxidative factors regulation

To study to which extent redox dysregulation is related to *APOE* ε4, CIND-AD participants were dichotomized according to the presence of the ε4 allele (*APOE* $\varepsilon4^+$; n = 10) or not (*APOE* $\varepsilon4^-$; n = 5). As shown in our previous work,[28] in cEV subpopulations, the mean size was lower in *APOE* $\varepsilon4^+$ carriers than noncarriers (data not shown). Our findings demonstrate that the observed changes in TAC levels, cEV apoD, and cEV apoJ/D ratio is *APOE* ε4-dependant (Figure 4A, C, and E). However, the presence of *APOE* ε4 variant did not affect cEVs protein carbonyls and plasma apoD levels (Figure 4B and D). To further confirm the influence of *APOE* ε4 variant we performed a linear regression analysis (Table 2). The univariate analysis supports a causal relationship between the studied parameters (as independent variables) and *APOE* ε4 variant (as dependent variable). Our results show a strong positive correlation between the ε4 allele and cEV apoD concentration whereas TAC and cEV apoJ/D ratio levels were negatively associated with *APOE* ε4 presence.

The areas under the ROC curves were measured to evaluate the discrimination capacity of the *APOE* ε4 variant (Figure 5 and Table S4 in supporting information). When *APOE* $\varepsilon4^+$ participants were compared to controls, ROC analysis revealed excellent AUC values for TAC (0.8214 ± 0.0043; $P = .0043$) and cEV apoD levels (0.8238 ± 0.076; $P = .0041$; Figure 5B and E), whereas moderate diagnostic accuracy was noted for apoJ/D ratio (0.7571 ± 0.091; $P = .022$) and cEV protein carbonyls (0.75 ± 0.094; $P = .027$; Figure 5H and K). In contrast, differentiation power did not reach statistical significance when *APOE* $\varepsilon4^-$ participants were compared to controls, except for cEVs protein carbonyls (Figure 5C, F, I, and L). Similarly, poor discrimination capacity was noted when *APOE* ε4+ and ε4− participants were merged and compared to controls (Figure 5A, D, G and J).

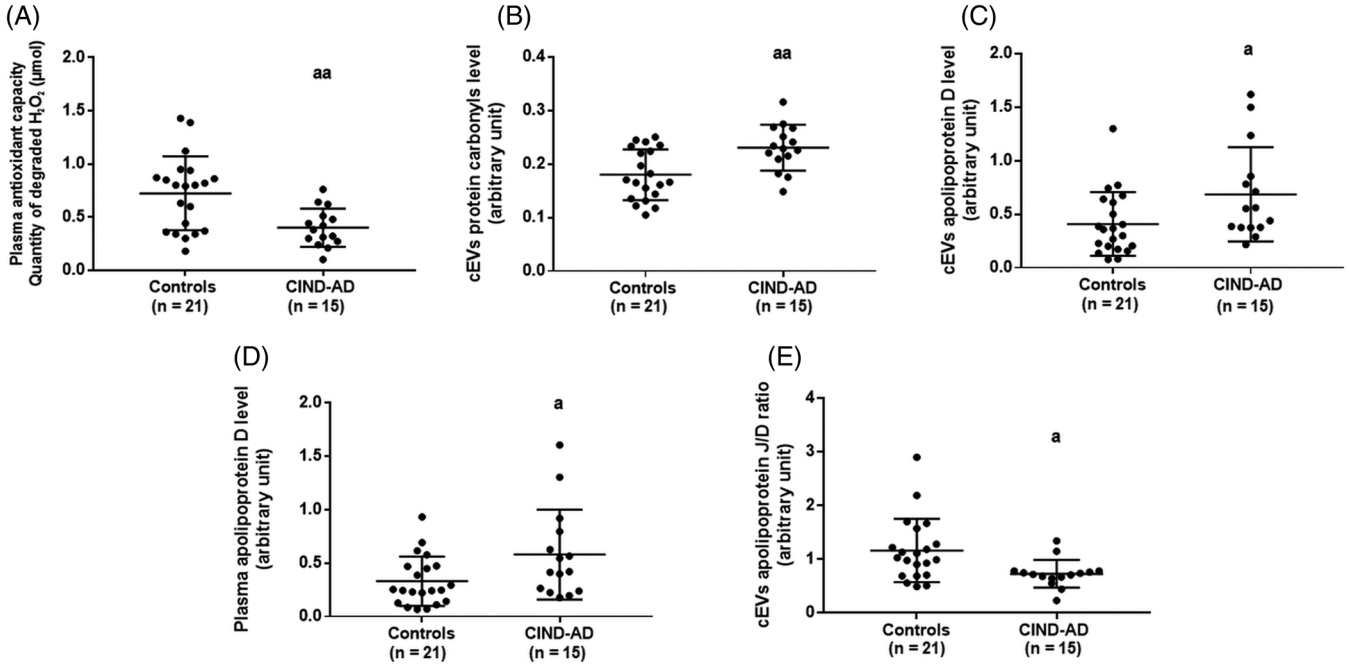

FIGURE 2 Plasma and circulating extracellular vesicle (cEV) levels of oxidative factors. A, Plasma TAC, (B) cEVs protein carbonyls, (C) cEVs apolipoprotein D, (D) plasma apolipoprotein D, (E) cEVs apolipoprotein J/D ratio (N = 36, including 21 controls and 15 CIND-AD). Each point represents the ratio of marker band intensity and the corresponding total protein intensity stained with the Coomassie blue. Statistical analysis was performed using the nonparametric Mann-Whitney test for (A), (C), (D), and (E) parameters and parametric student *t* test for (B) with a, $P < .05$ and aa, $P < .01$ versus controls

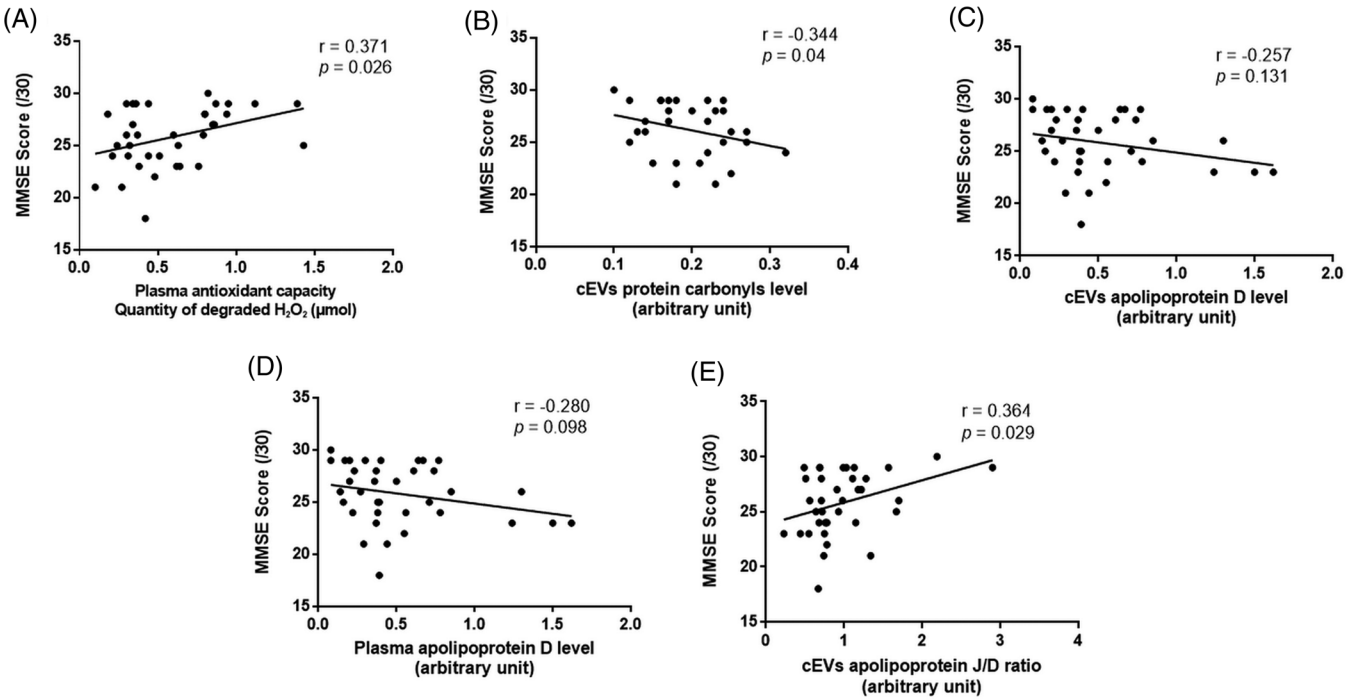

FIGURE 3 Linear regression analysis between the cognitive performances and the studied markers: (A) plasma total antioxidant capacity, (B) circulating extracellular vesicle (cEV) protein carbonyls, (C) cEV apolipoprotein D, (D) plasma apolipoprotein D, (E) cEV apolipoprotein J/D ratio (N = 36, including 21 controls and 15 cognitively impaired, no dementia-Alzheimer's disease). Abbreviations: MMSE, Mini-Mental State Examination; r, correlation coefficient; *p*, significance. Statistical analysis was performed using the correlation coefficient (Pearson r) and *P*-values were determined using Pearson correlation

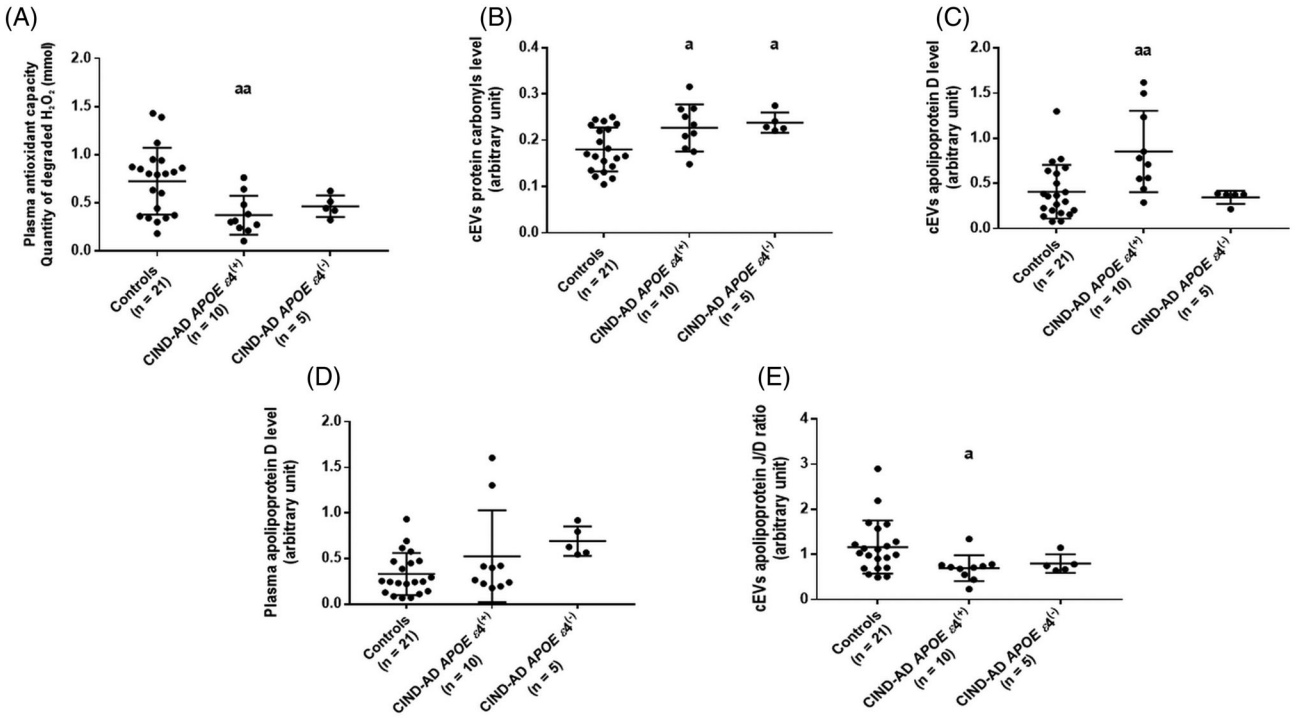

FIGURE 4 Plasma and circulating extracellular vesicle (cEV) levels of oxidative factors according to *APOE* isoform: (A) plasma total antioxidant capacity, (B) cEV protein carbonyls, (C) cEV apolipoprotein D, (D) plasma apolipoprotein D, (E) cEV apolipoprotein J/D ratio (N = 36, including 21 controls, 10 CIND-AD *APOE* $\varepsilon4^+$, and 5 CIND-AD *APOE* $\varepsilon4^-$). Abbreviations: CIND, cognitively impaired, no dementia; AD, Alzheimer's disease; *APOE*, apolipoprotein E; (+), ε4 carriers; (−), ε4 non-carriers. Each point represents the ratio of marker band intensity and the corresponding total protein intensity stained with the Coomassie blue. Statistical analysis was performed using the nonparametric Kruskal-Wallis test followed by Dunn test for (A), (C), (D), and (E) parameters and the one-way analysis of variance (ANOVA) followed by the Dunnett post hoc test for (B). a, $P < .05$ and aa, $P < .01$ versus controls

TABLE 2 Univariate correlation of oxidative stress factors and *APOE* ε4 isoform in the study population

		Plasma TAC	cEVs PCs	cEVs apoD	Plasma apoD	cEVs apoJ/D
APOE ε4	r	−.420	.312	.542	.166	−.347
	p	.011*	.068	.001**	.333	.038*
	N	36	36	36	36	36

Abbreviations: apoD, apolipoprotein D; *APOE*, apolipoprotein E; apoJ/D, apolipoprotein J/apolipoprotein D; cEVs, circulating extracellular vesicles; N, number of participants; *p*, significance (2-tailed); PCs, protein carbonyls; r, Pearson correlation coefficient; TAC, total antioxidant capacity; ε4, *APOE* isoform.
*$P < .05$.
**$P < .01$.

4 | DISCUSSION

Oxidative stress is a unifying paradigm of functional and structural brain changes observed in AD. In conjunction with a state of oxidative stress, *APOE* ε4 is assumed to stimulate oxidative damage compared to other apoE isoforms.[15,16,35] Considering that oxidative stress is one of the early events in the pathophysiology of AD, the present paper aimed to identify a redox signature that can predict the progression of pre-demented people to AD and to investigate the role of *APOE* ε4 in impaired redox homeostasis.

There is currently great interest in the use of peripheral EVs as a reliable screening tool in AD diagnosis. Most clinical research studies investigated inflammatory and pathogenic proteins, neurotrophic factors, glycation products, and their precursors as well as miRNA pool contained in cEVs.[26–30] However, no study has targeted oxidative stress markers in cEV cargo or compared their diagnostic sensitivity to plasma. In this context, our study analyzed a profile of several antioxidant defense systems in cEVs and in plasma that could predict the conversion of CIND participants to AD.

Apolipoproteins are closely linked to neurodegenerative pathologies and recent reports support their use as novel biomarkers for AD.[19,36,37] In the brain, apoE, apoJ, and apoD are the most abundant apolipoproteins and the most studied in the normal aging process as well as in AD. Our study evidences the presence of apoE,

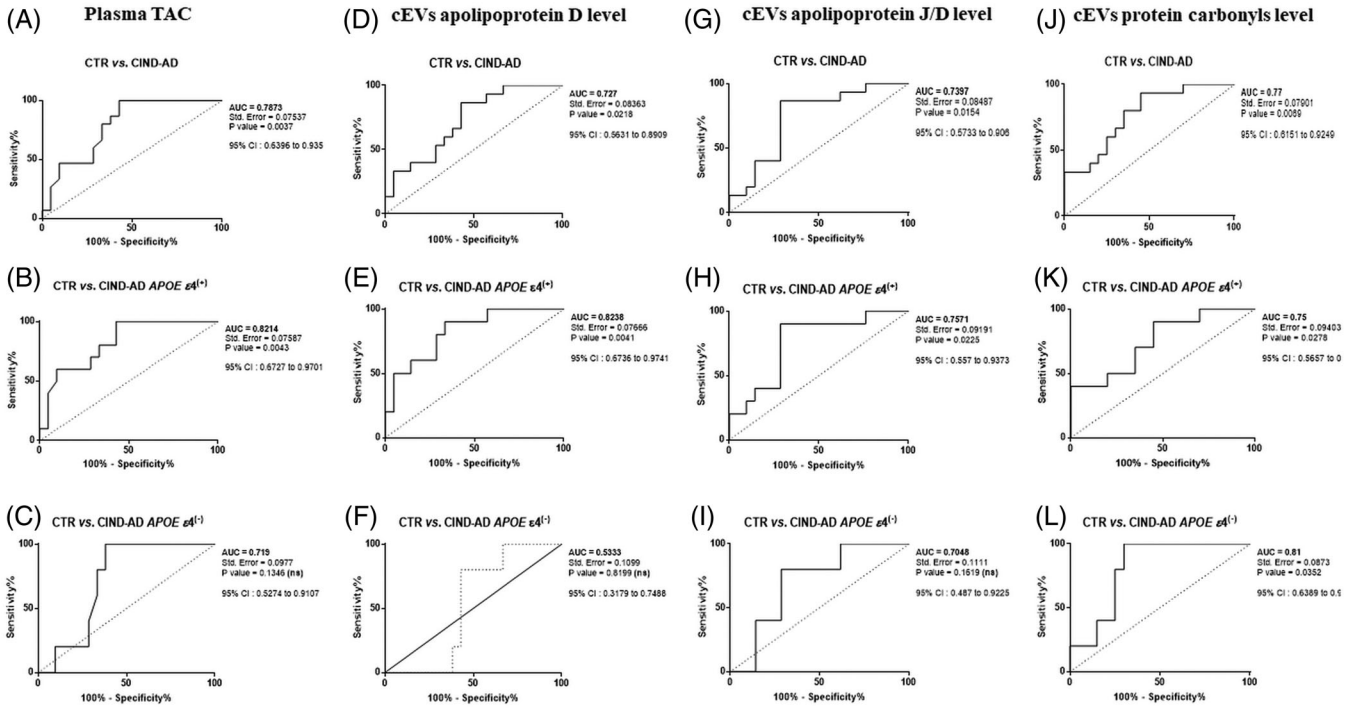

FIGURE 5 Receiver operating characteristic (ROC) curve analysis according to apolipoprotein E (*APOE*) isoform. The plot represents the performance of plasma and circulating extracellular vesicle (cEV) oxidative factors to discriminate controls from total CIND-AD, CIND-AD *APOE* $\varepsilon4^+$, and CIND-AD *APOE* $\varepsilon4^-$ participants: (A, B, C) plasma total antioxidant capacity, (D, E, F) cEV apolipoprotein D, (G, H, I) cEV apolipoprotein J/D ratio, (J, K, L) cEV protein carbonyls level. AUC, area under the curve; Std, standard; 95% CI, 95% confidence intervals; ns, not significant

apoJ, and apoD in cEVs but only cEVs apoD and to a lesser extent plasma apoD levels were higher in CIND-AD cases compared to controls. Several studies have demonstrated higher apoD levels in the cortex, hippocampus, and cerebrospinal fluid of AD patients[34,38] as well as in brain tissue of AD transgenic mice.[39] Previous in vitro and *post mortem* studies indicated that apoD acts as an antioxidant and neuroprotective molecule in age-related degeneration and neurodegenerative disease.[40] For instance, apoD levels were increased under stressful conditions such as brain injury.[41] Moreover, the protection of SH-SY5Y neuron from paraquat-triggered oxidative stress was attributed to the presence of apoD in astroglial-derived EVs.[42] Furthermore, during AD, increased apoD expression in multiple brain compartments is believed to counteract oxidative stress induced–damage.[43]

In the present study, the increase of apoD levels was consistent with reduced TAC capacity and increased cEV protein carbonyls content suggesting that its upregulation during early-AD stages is an active mechanism to overcome a gradual decline in antioxidant systems efficiency and escalating oxidative stress burden.

Several studies evaluated the total antioxidant status using similar analytical methods that differ sometimes in terms of reaction mechanisms. In line with our findings, a reduction in serum and plasma TAC was observed in MCI and AD patients.[19,44] Previous studies also reported impaired protein carbonyls levels in both plasma and brain of patients with early- and late-AD stages.[19,45,46] However, it is important to note that our study is the first to detect protein carbonyls and apolipoproteins in cEVs, along with plasma, and to analyze their content in preclinical AD patients.

Because peripheral oxidative stress markers could reflect systemic variations rather than CNS-related disorders, we assessed the relationship between the studied factors and cognitive performance. Interestingly, TAC and cEV apoJ/D ratio were positively correlated to MMSE cognitive scores suggesting that these antioxidant systems are important to sustain adequate brain function and normal cognitive abilities. Meanwhile, elevated cEV protein carbonyls levels were associated with altered neurocognitive performance. As cEVs can shuttle from CNS to peripheral circulation through the BBB,[24,47] it is suggested that they might reflect the brain pathological processes. Interestingly, astrocytes and glial cell markers were detected in our isolated cEVs providing further evidence that some cEVs are of brain origin. To date, few techniques were developed to isolate plasma CNS-enriched EVs, essentially by targeting neuronal-EV surface marker proteins. Nonetheless, poor fractions were obtained due to the lack of neural tissue-specificity of these markers (such as L1CAM).[48] Moreover, the study of total cEV cargo might be more reliable than nEVs considering the complex pathophysiology of AD and the critical role of metabolic disorders in increased AD risk. Altogether, our data support the utility of cEVs in AD diagnosis and demonstrate their higher sensibility to detect systemic variations compared to plasma, which is likely due to the efficient protection of cEVs' molecular components by their rich lipid membrane layer acting as an efficient biological shield.

The pathological linkage between *APOE* ε4-induced oxidative stress and AD risk is well established.[6,15–17] When CIND-AD cases were classified by their *APOE* genotype, the significant differences were more pronounced in the *APOE* ε4$^+$ group. ROC and linear regression analysis showed that the observed changes in TAC, cEV apoD, and cEV apoJ/D levels were assigned to the presence of *APOE* ε4 isoform, whereas cEV protein carbonyls content was slightly affected by the ε4 allele. Similarly, reduced serum and plasma levels of TAC were reported to exacerbate the risk of AD in *APOE* ε4 individuals.[44,49] Besides, the toxic features of apoE4 protein appear to trigger a variety of oxidative pathways leading to carbonylation of protein and apolipoprotein dysregulation.[50] Consequently, the reduced antioxidant defense in *APOE* ε4 carriers may contribute to the early pathological cascade of neurodegeneration. Our study is the first to establish this relationship in cEVs, which may help to explain the *APOE* ε4-induced increase of oxidative stress over the course of the disease.

Overall, the present work shows that CIND-AD *APOE* ε4 carriers display differential regulation of several oxidative markers that impact brain homeostasis and illustrate the extended effect of this allele on multiple oxidative patterns beyond its influence on the clinical phenotype of AD.

The limitations of the present study should be regarded. Future investigations should include correlations with neuroimaging and/or cerebrospinal fluid data, besides cognitive tests, to confirm the exclusive relationship with brain malfunction. In addition, the analyzed parameters do not reflect the full spectrum of oxidative markers, which entails a thorough screening of other stress candidates in upcoming research. The sample size is adequate for statistical analysis in case-control studies but not large enough for epidemiological considerations, which implies future larger sample size cohorts to validate our findings. It is also important to note the susceptibility of the analyses to confounders and to some unobserved variables that could affect the observed signals. Another potential limitation of this study is the lack of multiple test correction and the possible impact of specific genetic ancestries. Future studies should also explore these interactions across similar and various biomarkers, which may increase the specificity of their predictive values.

5 | CONCLUSIONS

Taking together, our results demonstrate that, (1) cEVs' redox signature is more relevant than plasma for reflecting specific brain and systemic changes in early AD onset, (2) the pathological implication of *APOE* ε4 allele is mainly due to a gain of toxic functions combined with a loss of protective ones, (3) future studies should consider integrating *APOE* genotyping in oxidative stress biomarkers identification, and (4) the heterogeneity of preclinical patients calls for a shift in paradigm where targeted diagnosis should be developed for specific AD subpopulations that share similar pathological or genetic properties.

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CONFLICTS OF INTEREST

The authors report that they have no conflicts of interest to disclose.

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