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Reelin Plasma Levels Identify Cognitive Decline in Alcohol Use Disorder Patients During Early Abstinence: The Influence of APOE4 Expression

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ABSTRACT

Background: Apolipoprotein E (APOE)-4 isoform, reelin, and clusterin share very–low-density liporeceptor and apolipoprotein E receptor 2 receptors and are related to cognition in neuropsychiatric disorders. These proteins are expressed in plasma and brain, but studies involving plasma expression and cognition are scarce.

Methods: We studied the peripheral expression (plasma and peripheral blood mononuclear cells) of these proteins in 24 middle-aged patients with alcohol use disorder (AUD) diagnosed at 4 to 12 weeks of abstinence (t = 0) and 34 controls. Cognition was assessed using the Test of Detection of Cognitive Impairment in Alcoholism. In a follow-up study (t = 1), we measured reelin levels and evaluated cognitive improvement at 6 months of abstinence.

Results: APOE4 isoform was present in 37.5% and 58.8% of patients and controls, respectively, reaching similar plasma levels in ε4 carriers regardless of whether they were patients with AUD or controls. Plasma reelin and clusterin were higher in the AUD group, and reelin levels peaked in patients expressing APOE4 ($P < .05$, $\eta^2 = 0.09$), who showed reduced very–low-density liporeceptor and apolipoprotein E receptor 2 expression in peripheral blood mononuclear cells. APOE4 had a negative effect on memory/learning mainly in the AUD group ($P < .01$, $\eta^2 = 0.15$). Multivariate logistic regression analyses identified plasma reelin as a good indicator of AUD cognitive impairment at t = 0. At t = 1, patients with AUD showed lower reelin levels vs controls along with some cognitive improvement.

Conclusions: Reelin plasma levels are elevated during early abstinence in patients with AUD who express the APOE4 isoform, identifying cognitive deterioration to a great extent, and it may participate as a homeostatic signal for cognitive recovery in the long term.

Keywords: Alcohol Use Disorder, APOE4, clusterin, cognition, reelin

Significance Statement

Identifying biological markers for tracking the progression of neuropsychiatric disorders is crucial for supporting vulnerable patients. Our study discovered Reelin, which was elevated during early abstinence (4–12 weeks) in patients with alcohol use disorder (AUD) undergoing outpatient treatment. Plasma reelin was higher in the AUD group compared with controls, especially among patients with AUD expressing plasma APOE4, an aberrant isoform of APOE whose coding gen (ε4 allele) was linked to neuroinflammation and cognitive impairment. Plasma APOE4 did not differ between AUD and control ε4-carriers. However, reelin peaked in patients with AUD expressing APOE4, with no changes in control carriers, and it correlated with worse cognition during early abstinence. Follow-up studies showed lower reelin levels in the AUD group after 6 months of abstinence, accompanied by cognitive improvements. These findings suggest that reelin could identify AUD cognitive impairment during early abstinence and may act as a homeostatic signal involved in long-term cognitive amelioration.

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INTRODUCTION

Alcohol use disorder (AUD) is considered a public health issue characterized by a problematic pattern of alcohol consumption that leads to induced brain damage and cognitive impairment (Wollenweber et al., 2014; Aharonovich et al., 2018; Visontay et al., 2021). The cognitive deterioration profile in patients with AUD is on a spectrum (Hayes et al., 2016) ranging from lower cognitive decline (Neafsey and Collins, 2011; Sabia et al., 2014) to dementia (Visontay et al., 2021). The cognitive domains more severely affected include visuospatial abilities, memory, and executive functioning (EF) (Hayes et al., 2016; Sachdeva et al., 2016).

The endogenous mechanisms that mediate cognitive decline in AUD are still unknown. One widely studied biomarker related to cognition is the presence of the ε4 allele in the Apolipoprotein (Apo)E gene, which codes for the APOE4 isoform, considered dysfunctional in older adults (reviewed in Hauser et al., 2011). APOE4 is expressed in a subset of the population (ApoE-ε4 carriers) who carry 1 or more copies of the allele (i.e., ε3/4 heterozygotes and ε4/4homozygotes). APOE-ε4inheritance is one of the most accepted genetic risk factors for developing late-onset Alzheimer disease (AD) (Schmechel et al., 1993; Riedel et al., 2016; Serrano-Pozo et al., 2021) and has been related to neuroinflammation (Kloske and Wilcock, 2020; Duro et al., 2022) and the emergence of cognitive decline (Reivang et al., 2010; Montagne et al., 2020). Recent studies have identified a higher vulnerability of ApoE-ε4 carriers to the toxic effects of alcohol, even with small consumptions (Slayday et al., 2021), whereas other studies associated ApoE-ε4 with the risk of dementia independent of alcohol consumption (Heffernan et al., 2016). The impact of APOE4 presence on AUD remains largely unstudied, and most studies examining APOE4 and cognition focus on genetics, providing limited information on the presence of APOE4 and its related signaling molecules in peripheral tissues.

The APOE protein shares the very–low-density liporeceptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2) with clusterin (APOJ) and reelin (Lane-Donovan and Herz, 2017; Dlugosz and Nimpf, 2018), which have also been related with cognition. While APOE4and clusterin have been associated to cognitive impairment, reelin plays a pivotal role in brain development, cortical synaptic plasticity (Tissir and Goffinet, 2003; Herzand Chen, 2006), and improved cognition (Stranahan et al., 2013; Ishii et al., 2016).

Despite some evidence suggesting that these molecules (APOE4, reelin, and clusterin), which share common receptors, may modulate cognition in several neuropsychological disorders, there is still scarce information about the role of peripheral (vs central) APOE4, reelin, and clusterin in cognition, specifically in patients diagnosed with AUD. In the field of alcohol abuse, several challenges exist: (1) the lack of uniformity in the assessment of cognitive impairment in AUD without a specific neuropsychological test for this pathology; (2) most studies on APOE4 focus on genetics with no measurement of APOE4 isoform levels in plasma, and there is scarce comprehension about a possible role of peripheral APOE4, reelin, or clusterin on AUD-induced cognitive deterioration. Here, we used a specifically validated cognitive test for AUD-diagnosed patients (Jurado-Barba et al., 2017) and evaluated the APOE4 isoform, reelin, and clusterin in plasma together with their VLDLR and ApoER2 receptors in peripheral blood mononuclear cells (PBMCs), studying their association with cognition during early abstinence in a young/middle-aged population.

METHODS

Study Participants

A total 76 White Caucasian patients from early- to late-middle adulthood were recruited (see flowchart, Fig. 1) and divided into 2 groups: (1) AUD group: 39 abstinent patients recruited from an outpatient "Alcohol Programme" (Methods in supplementary Information 1.1), and (2) control group: 37 healthy patients with no history of drug abuse who were recruited from the general population (random sampling of normal adults; supplementary Information 1.1). After corroborating the eligibility for the study, 24 patients (17 men and 7 women) were included in the AUD group and 34 (16 men and 18 women) in the control group.

Inclusion Criteria

Inclusion criteria were aged between 18 and 65 years, AUD diagnosis based on DSM-5 criteria (APA, 2014), and alcohol abstinence for at least 4 weeks before testing. Abstinence was monitored through exhaled breath controls during hospital visits (supplementary Information 1.2).

Exclusion Criteria

Exclusion criteria were history of abuse or dependence on substances other than tobacco (including alcohol in the control group); psychiatric comorbidity or concomitant psychological disorder; mild-moderate to severe psychological symptoms; chronic medical condition, liver disease (chronic hepatitis, cirrhosis, or liver cancer), or infectious diseases (HIV infection and/or acute hepatitis); and chronic use of anti-inflammatory medication.

See clinical and psychological assessment methodology used to ensure compliance with inclusion/exclusion criteria in supplementary Information 1.2.

Alcohol Abuse Variables and Liver Status Parameters

A semi-structured interview was conducted for both groups to gather information on alcohol use history (supplemental Table 1).

The liver status was evaluated in the AUD group by analyzing alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and bilirubin levels in plasma, which is standard clinical practice.

Neuropsychological Testing

Participants were assessed by "Test of detection of cognitive impairment in alcoholism" (TEDCA), specifically validated in patients with AUD (Jurado-Barba et al., 2017). This test provides a snapshot of cognitive functioning (General Cognitive Functioning [GCF]) based on a compendium of tests assessing 3 specific cognitive domains: visuospatial cognition, memory/learning, and EF (supplementary Information 1.3 and Table 2).

Sample Collection and Processing

Blood extractions were performed in the morning (8:00–10:00 AM) after 8 to 12 hours of fasting. Blood was collected into 10-mL BD Vacutainer tubes with K2-EDTA anticoagulant (BD, Franklin Lakes, NJ, USA) and processed immediately to obtain plasma and PBMCs (supplementary Information 1.4). Samples were coded and stored at −80 °C.

Plasma APOE4 Isoform Determination

APOE4 plasma levels were determined using the e4Quant patented technique (Biocross S.L.), which is based on turbidimetry

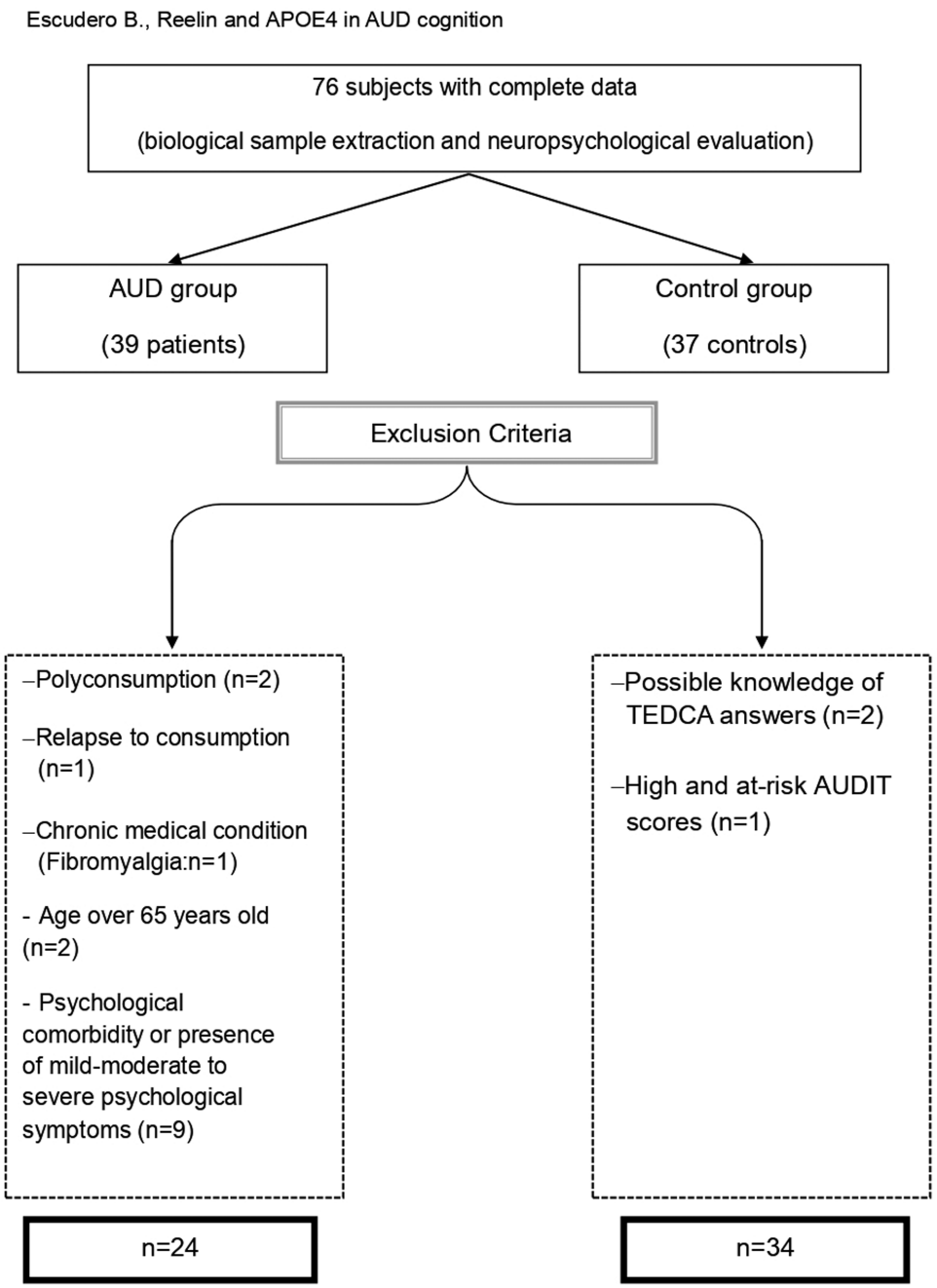

Figure 1. Flowchart on sample recruitment. Participants were divided into 2 experimental groups: alcohol use disorder (AUD) group and control group. Based on the exclusion criteria previously established for the study, the final selected sample is established. AUDIT, Alcohol Use Disorders Identification Test; TEDCA, test of detection of cognitive impairment in alcoholism.

(Calero et al., 2018). e4Quant is a validated method specifically designed to detect and quantify APOE4 in human plasma, excluding APOE3 or APOE2 isoforms (supplementary Information 1.5).

Reelin, Clusterin, VLDLR, and ApoER2 Detection

Reelin and clusterin were determined in plasma, while VLDLR and ApoER2 in PBMCs were determined using commercially available enzyme-linked immunosorbent assay kits and following the manufacturer's instructions (supplementary Information 1.6).

Follow-Up Study

Patients were evaluated at recruitment for the study (t=0; 4–12 weeks of abstinence) and at 6 months of abstinence (t=1) (details in supplementary Information 1.7).

Statistical Analyses

Comparisons between AUD and control groups were analyzed by Fisher exact test, chi-square test, or Student t test according to the statistical assumptions for each test. Results are shown as number and percentage of patients (N [%]) or mean (SD).

ANCOVA with group, APOE4 presence, and time as factors (based on each analysis) were performed considering the assumptions of covariate independence and homoscedasticity by Levene test. Covariates for all ANCOVAs were age and education. ANCOVAs are shown as follows: F-statistic (factor/error degrees of freedom), *P* value, and effect size partial eta-squared η^2. The Bonferroni post hoc test was conducted when appropriate. Figures are displayed as scatterplots, with mean (SD).

The relationship between biomarkers and cognition/alcohol abuse/liver variables was studied using Spearman rank coefficients (r) (adjusted R^2). To account for multiple testing in the correlations, *P* values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) at 1%. This correction helps limit false-positive findings among all statistically significant findings to a certain threshold.

Hierarchical logistic regression models were performed to determine the contribution of each biomarker (reelin, APOE4, clusterin, VLDLR, ApoER2) in explaining the GCF deficit. We employed a stepwise approach by utilizing models for GCF that progressively controlled for potential confounding factors, including age and educational level. Model 1 included all biomarkers (reelin, APOE4, clusterin, VLDLR, ApoER2), Model 2 included age, and Model 3 further incorporated educational level. Reelin was transformed into standard Z scores for a better interpretation of the odds ratios because the transformation did not affect the Wald values and corresponding *P* values. The observations in our data set were independent; the Hosmer-Lemeshow assumption was met, and there was no multicollinearity. A receiving operating characteristic curve analysis was performed for GCF to determine the thresholds at which reelin could be considered a risk factor.

Statistical analyses were performed through IBM SPSS Statistical Version 25.0 software (IBM, Armonk, NY, USA) and GraphPad Prism version 8.00 (GraphPad Software, Inc., San Diego, CA, USA). $P < .05$ was considered statistically significant.

RESULTS

Sample Demographics

Sociodemographic comparisons between AUD and control groups are presented in Table 1, together with medication use. There were no significant differences in potential confounding variables (education level, current work status, etc.) except for age ($P < .01$). Both groups had a body mass index within the normal range, healthy cholesterol levels, and an equal number of current smokers. The most commonly used psychiatric medication in the AUD group was disulfiram, followed by antidepressants. No participants in the control group received psychiatric medication.

Alcohol Abuse and Liver Status Results in the AUD group

Alcohol abuse outcomes are summarized in supplementary Table 3 and supplementary Information 2.1. Briefly, the mean duration of abstinence at recruitment (t = 0) was 44.25 (SD = 23.24) days and the length of alcohol abuse since the last relapse was a mean 39.54 (SD = 19.69) weeks.

Results of liver status are shown in supplementary Table S4 (supplementary Information 2.2). The mean values of ALT, AST, GGT, ALP, and bilirubin fell within normal ranges.

Neuropsychological Findings

Cognitive data from AUD and control groups are presented in Table 2. Patients with AUD showed significant lower cognitive performance in all domains and GCF ($P < .01$). The mean of GFC scores in

Table 1. Sociodemographic and Pharmacological Variables in AUD and Control Groups

Variables		AUD (n = 24)	Control (n = 34)	*P*
Age, mean (SD)	Years old	49.17 (6.52)	37.41 (12.61)	**0.00**[a]
BMI, mean (SD)	kg/m^2	26.68 (5.20)	24.43 (3.90)	0.06[a]
Sex, n (%)	Women	7 (29.2)	18 (52.9)	0.12[b]
	Men	17 (70.8)	16 (47.1)	
Education, n (%)	No high school degree	2 (8.3)	—	0.07[c]
	High school degree	7 (29.2)	5 (14.7)	
	College degree	15 (62.5)	29 (85.3)	
Current work status, n (%)	Employed	19 (79.2)	32 (94.1)	0.11[b]
	Unemployed	5 (20.8)	2 (5.9)	
Current smoking status, n (%)	Yes	16 (66.7)	16 (47.1)	0.33[c]
	Former	2 (8.3)	4 (11.8)	
	No	6 (25.0)	14 (41.2)	
Psychiatric medication use, n (%)	Antidepressants	11 (45.8)	No use	—
	Anxiolytics	6 (25.0)	No use	—
	Anticonvulsants	9 (37.5)	No use	—
	Antipsychotics	3 (12.5)	No use	—
	Disulfiram	20 (83.3)	No use	—

Abbreviations: AUD, alcohol use disorder; BMI, body mass index. The significant values ($P < .05$) are denoted by bold entries in the table.
[a]*P* value from Student t test.
[b]*P* value from Fisher exact test.
[c]*P* value from chi-square test.

Table 2. Cognitive scores in TEDCA in the AUD and control groups

Cognitive Domains		GCF	Visuospatial Cognition	Memory /Learning	EF
Direct scores [mean (SD)]	AUD (n=24)	11.75 (3.27)	4.62 (1.31)	3.04 (1.46)	4.04 (1.49)
	Control (n=34)	14.85 (1.94)	5.38 (0.78)	4.79 (0.98)	4.74 (1.12)
P		**0.00**[a]	**0.01**[a]	**0.00**[a]	**0.04**[a]

Abbreviations: AUD, alcohol use disorder; EF, executive functioning; GCF, general cognitive functioning. The maximum score in GCF is 18 and cognitive impairment is established at a cutoff point ≤10.5. The significant values ($P < 0.05$) are denoted by bold entries in the table.
[a]*P* value from Student *t* test.

Table 3. Peripheral APOE4 Expression and Levels, Reelin, Clusterin, VLDLR, and ApoER2 in AUD and Control Groups

Variables		AUD (n=24)	Control (n=34)	P
Plasma APOE4 expression n (%)	Yes	9 (37.50)	20 (58.80)	0.11[a]
	No	15 (62.50)	14 (41.20)	
APOE4 levels (ApoE-ε4carriers) (μg/mL), mean (SD)		11.28 (9.30)	11.08 (6.40)	0.95[b]
Reelin levels (ng/mL), mean (SD)		0.42 (0.20)	0.30 (1.59)	**0.02**[b]
Clusterin levels (μg/mL), mean (SD)		46.65 (22.89)	30.72 (20.89)	**0.01**[b]
VLDLR levels (ng/mL total protein), mean (SD)		4.14 (2.74)	5.32 (3.49)	0.17[b]
ApoER2 levels (ng/mL total protein), mean (SD)[c]		3.36 (2.30)	3.20 (2.03)	0.78[b]

Abbreviations: n, total of cases; SD, standard deviation. APOE4, Reelin and Clusterin are determined in plasma. VLDLR and ApoER2 are determined in peripheral blood mononuclear cells (PBMCs). The significant values ($P < .05$) are denoted by bold entries in the table. In those expressing plasma APOE4 (0 values are not considered).
[a]*P* value from chi-square test,
[b]*P* value from Student *t* test.
[c]n=23 due to the presence of an outlier in the AUD group.

the AUD group was above the cutoff point for TEDCA to consider impairment (cutoff ≤10.5; supplementary Information 1.3), and the 29.2% of the AUD group fulfilled the criteria for GCF deficit.

Status of Biomarkers

Biochemical results are presented in Table 3. The e4Quant technique showed the number and percentage of patients with AUD and controls expressing the plasma APOE4 isoform, as well as the amount of APOE4 expressed in plasma (if presence). There was a higher percentage of patients with plasma APOE4 presence in the randomly recruited control group (58.80%) vs the AUD group (37.50%) without significant differences ($P = .11$, chi square test) (Table 3). When considering only Apo-ε4 carriers, we observed a similar amount of APOE4 in plasma for both experimental groups ($P = .95$, Student *t* test), suggesting no apparent interference of alcohol in the quantity of plasma APOE4.

Regarding reelin levels (Table 3), a 2-way ANCOVA controlling for covariates showed a main effect of group ($F_{(1,52)} = 8.12$, $p = .006$, $\eta^2 = 0.135$) (with higher levels in the AUD group), a main effect of *APOE4* ($F_{(1,52)} = 6.63$, $P = .01$, $\eta^2 = 0.113$) (raised levels in ApoE-ε4 carriers), and an interaction between group*APOE4 ($F_{(1,52)} = 5.37$, $P = .02$, $\eta^2 = 0.094$] (Fig. 2A). Bonferroni post hoc analyses revealed a difference in reelin levels between patients with AUD expressing and not expressing the APOE4 ($t = 3.699$; $P < .01$), which did not appear in controls, so that reelin levels were higher when the AUD patient was an ApoE-ε4 carrier (see Fig. 2A).

Analyses of clusterin levels (Table 3) revealed a group effect ($F_{(1,52)} = 8.84$, $P = .004$, $\eta^2 = 0.145$) (with higher levels in the AUD group) and no effects of *APOE4* or interaction (2-way ANCOVA controlling for covariates; $P > .05$; data not shown). The presence of APOE4 or lack thereof did not have a significant influence on clusterin levels, although a trend could be observed (Fig. 2B).

We detected the presence of the receptors ApoER2 and VLDLR in PBMCs in both AUD and control groups (Table 3). Regarding ApoER2, a 2-way ANCOVA controlling for covariates revealed a main effect of *APOE4* ($F_{(1,51)} = 9.32$, $P = .004$, $\eta^2 = 0.155$) (lower levels in APOE4 carriers) and an interaction between group*APOE4 ($F_{(1,51)} = 4.14$, $P = .47$, $\eta^2 = 0.075$) (Fig. 2C). Post hoc analyses revealed a difference between APOE4 carriers vs noncarriers in the AUD group, which was not observed in the control group, suggesting that ApoER2 PBMCs levels may be lower in patients with AUD expressing APOE4 plasma levels. Regarding VLDLR, there was a main effect of group*A-*POE4* ($F_{(1,52)} = 4.21$, $P = .04$, $\eta^2 = 0.075$) (Fig. 2D), with the lowest levels observed in patients with AUD carrying APOE4.

Associations Between Biomarkers and Alcohol Abuse or Liver Status Variables

Spearman rank correlations between biomarkers and alcohol abuse variables showed a significant association between reelin plasma levels and the length of alcohol abuse since last relapse ($r = 0.864$, $P = .00$). No associations were found with the other markers shown in Table S3.

Reelin plasma levels did not correlate with the liver status parameters (Spearman rank correlation): ALT ($r = -0.162$; $P = .451$); AST ($r = 0.173$; $P = .418$); GGT ($r = 0.200$; $P = .350$); ALP ($r = 0.127$, $P = .583$); Bilirubin ($r = 0.327$, $P = .138$).

Effect of Biomarkers on Cognition in Patients With AUD

Correlations between cognition and biomarkers

In the AUD group, reelin showed significant negative correlations with GFC ($r = -0.74$, $P = .00$, $q = 0.00$) and EF ($r = -0.75$, $P = .00$,

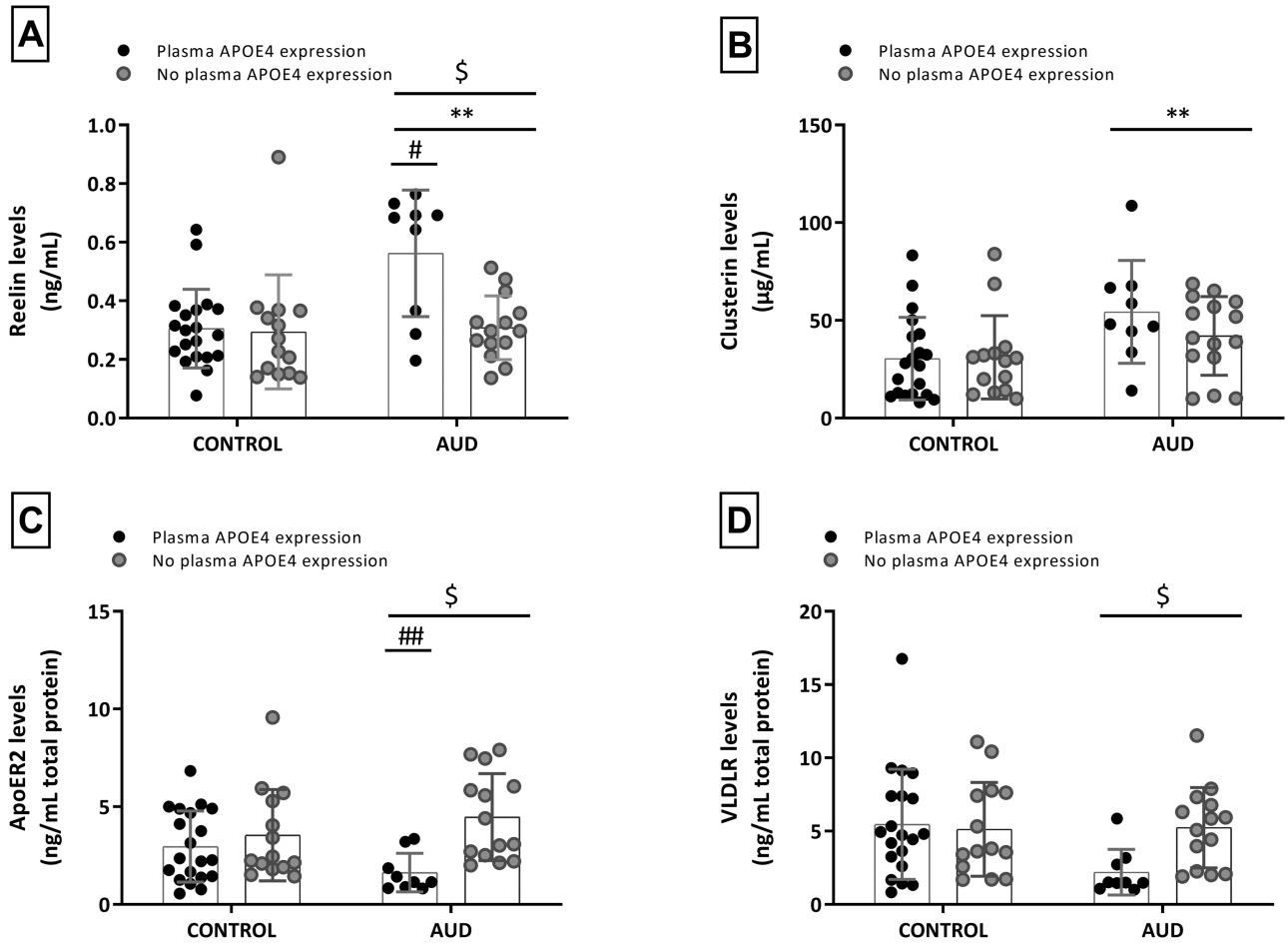

Figure 2. Levels of (A) reelin, (B) clusterin, (C) apolipoprotein E receptor 2 (ApoER2), and (D) very-low density liporeceptor (VLDLR) in the alcohol use disorder (AUD) and control groups based on ApoE4. Results are presented as mean (SD). Two-way ANCOVA with group and APOE4 as factors, controlling for age and education. Overall effect of group: $^{**}P<.01$; APOE4: $^{\#}P<.05$; $^{\#\#}P<.01$ and analysis of the interaction (group × APOE4): $^{\$}P<.05$. Bonferroni post hoc test after interaction.

q=0.00), which were maintained after adjustment by the FDR (supplementary Table 5; supplementary Information). The significant negative correlations of reelin and ApoER2 with memory/learning were lost after FDR adjustment, among others. In the control group, no significant correlations were found between cognitive measures and biomarkers (data not shown).Effect of the Presence of Plasma APOE4 (Carriers/Noncarriers) on Cognition

ANCOVA analyses controlling for age and education revealed: (A) group differences in all cognitive domains except visuospatial cognition, with lower scores observed in the AUD group (Fig. 3A–D) (GCF: $F_{(1,52)}=15.92$, $P=.000$, $\eta2=0.234$; memory/learning: $F_{(1,52)}=26.83$, $P=.000$, $\eta2=0.34$; EF: $F_{(1,52)}=4.53$, $P=.038$, $\eta2=0.08$); (B) the expression of plasma APOE4 (indicative of ApoE-ε4 carriers) influenced GCF, memory/learning, and EF, with carriers exhibiting lower scores (Fig. 3A,C,D) (GCF: $F_{(1,52)}=7.18$, $P=.01$, $\eta2=0.12$; memory/learning: $F_{(1,52)}=7.92$, $P=.007$, $\eta2=0.13$; EF: $F_{(1,52)}=4.25$, $P=.044$, $\eta2=0.076$) (no APOE4 effect on visuospatial cognition, $P>.05$); (C) an interaction between group and APOE4 presence on memory/learning (Fig. 3C) [F (1,52) =5.04, $P=.029$, $\eta2=0.088$], revealing worse performance in patients with AUD expressing APOE4 vs noncarriers (Bonferroni post hoc test; $P<.05$). APOE4 presence did not influence memory/learning in controls (Fig. 3C).Hierarchical Logistic Regression Models—Plasma reelin showed significance for GCF deficit up to the final model (Model 3: B=1.56, $P=.04$; Nagelkerke $R^2=0.51$; Hosmer and Lemeshow=0.63). The resulting equation was: [1.56 × reelin] + [0.01 ApoE4] + [−0.01 clusterin] + [0.23 × VLDLR] + [−0.31 ApoER2]+ [0.05 × age] + [0.52 × education] (Table 4). Among all biomarkers of the signaling pathway, reelin showed the highest odd ratio, meaning that is the marker whose increase in plasma better identifies the risk of cognitive impairment in the AUD group after controlling for remaining biological markers and all possible confounders.

A receiving operating characteristic curve was performed to explain GCF deficit because plasma reelin levels were shown to be significant in the logistic regression analysis (Figure 4). Specifically, the AUC for GCF was 0.90 ($P=.002$). The most optimal cutoff point for GCF was set at 0.36 (sensitivity: 1; specificity: 0.76) according to the maximum values of the Youden index (*J*).

Levels of Reelin and Cognition at 6-Month Follow-Up (t=1)

Given the strong association between reelin and cognitive impairment found in the AUD group, we conducted a 6-month follow-up study to check the evolution of reelin in patients with AUD and its possible influence on cognition.

Figure 5A shows the levels of reelin in the AUD and control groups according to the presence/absence of plasma APOE4 at t=0 (Fig. 5A, left) and at t=1 (Fig. 5A, right). Both time points were compared using 2-way ANCOVA analysis with age and education as covariates in each period. At t=0, we observed a main effect of

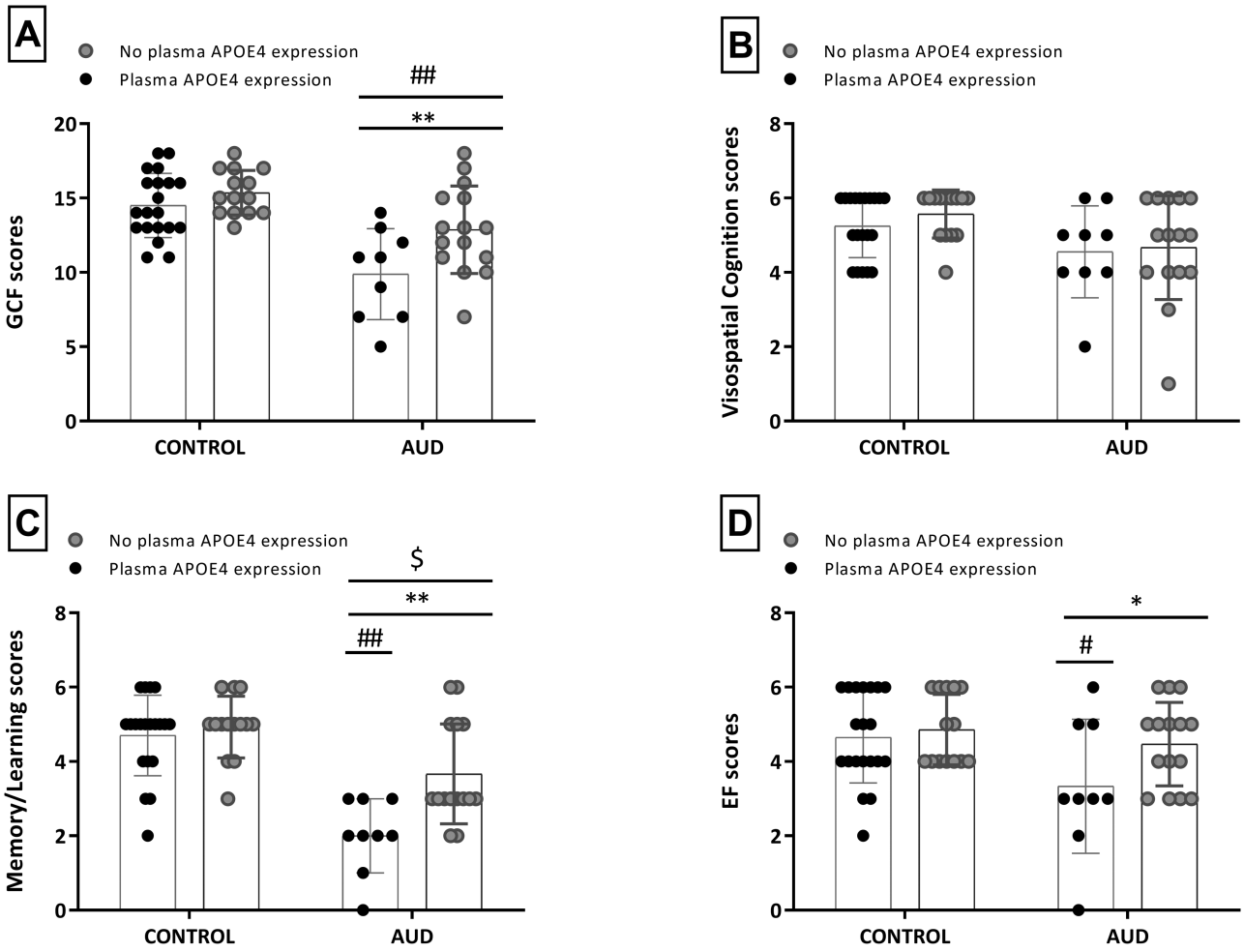

Figure 3. Effect of ApoE-ε4 on cognitive performance in the AUD and control groups. Two-way ANCOVA with group and APOE4 as factors, controlling for age and education. (A) GCF: General Cognitive Functioning, (B) visuospatial cognition, (C) memory/learning, (D) EF, Executive Functioning. Results as mean (SD). $^{**}P<.01$ and $^{*}P<.05$ denotes a main effect of group; $^{\#\#}P<.01$ and $^{\#}P<.05$ denotes a main effect of APOE4. Analysis of the interaction (group*APOE4) in (C): $^{\$}P<.05$. Bonferroni post hoc test after interaction.

Table 4. Hierarchical Logistic Regression Model: Association Between GCF Deficit and Biomarkers of the Reelin/APOE4 Pathway

	Variables Entered	B	SE	Wald	OR (95 % CI)	P
GCF	Reelin	1.56	0.77	4.12	4.77 (1.06–21.55)	**0.04**
	APOE4	0.01	0.09	0.01	1.01 (0.85–1.19)	0.93
	Clusterin	−0.01	0.03	0.17	0.99 (0.93–1.05)	0.68
	ApoER2	−0.31	0.88	0.12	0.74 (0.13–4.09)	0.73
	VLDLR	0.23	0.54	0.18	1.25 (0.43–3.63)	0.67
	Age	0.05	0.13	0.17	1.05 (0.82–1.36)	0.68
	Education	0.52	1.14	0.21	1.69 (0.18–15.73)	0.65

Abbreviations: CI, confidence interval; GCF, general cognitive functioning; OR, odds ratio; Wald, Wald test. Final multivariate logistic regression model including all biomarkers of the APOE/reelin pathway, controlled by age and educational level. Reelin data were transformed to Z scores. Significant values ($P < .05$) are denoted by bold entries in the table.

group, APOE4, and an interaction group*APOE4, with the highest reelin levels in ApoE- ε4 carriers within the AUD group, as mentioned in Fig. 2A (same analysis). At t=1, the AUD group had lower levels of reelin compared with the control group, as indicated by a significant group effect ($F_{(1,49)}=13.50$, $P=.001$, $\eta^2=0.216$). There were no significant effects of APOE4 or interactions ($P>.05$ n.s.) (Fig. 5A, right).

Figure 5B illustrates the cognitive evolution in the AUD group between t=0 and t=1. Repeated-measures 2-way ANCOVAs were performed with age and education as covariates. There was an interaction between group and time on GCF ($F_{(1,49)}=16.98$, $P=.01$, $\eta^2=0.121$) as well as in memory/learning ($F_{(1,49)}=6.35$, $P=.01$, $\eta^2=0.115$) and EF ($F_{(1,49)}=5.49$, $P=.02$, $\eta^2=0.10$), showing an improvement in cognition among the AUD group at t=1. In the control group, cognitive scores remained similar between t=0 and t=1 without a learning effect ($P>.05$, data not presented) (Fig. 5B).

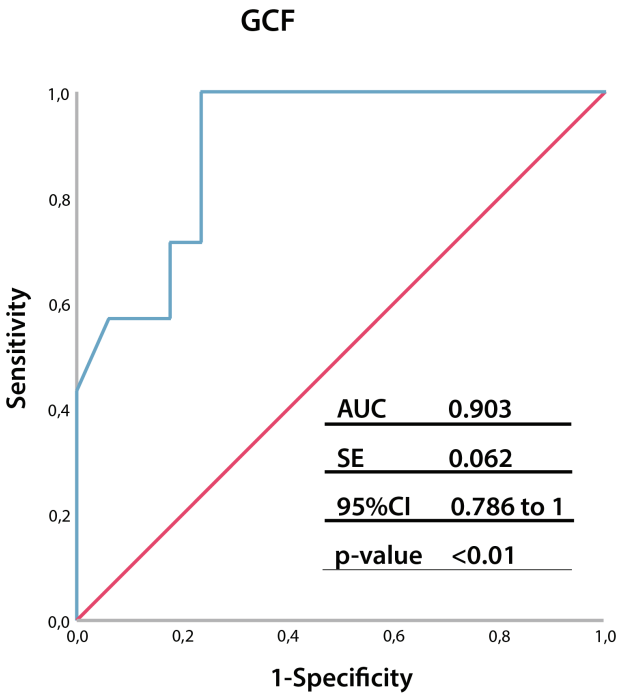

Figure 4. ROC analysis for reelin levels and GCF in the AUD group. ROC analysis for logistic regression models in the AUD group are shown, demonstrating the tradeoff between sensitivity (y-axis) and 1-specificity (x-axis). Both axes of the graph include values between 0 and 1 (0% to 100%). The line drawn from point 0.0 to the point 1.0 is called the reference diagonal, or the of nondiscrimination. AUC, area under the curve; CI, confidence interval; SE, standard error.

DISCUSSION

In this study we found plasma reelin as a biomarker that could identify patients with AUD with cognitive impairment during early abstinence. Plasma reelin was higher among patients with AUD expressing plasma APOE4 isoform, whereas being a ε4 carrier had no effects in the control group. Alterations in plasma clusterin and VLDLR and ApoER2 in PBMCs were found in the AUD group, unrelated to cognitive decline. In follow-up studies, plasma reelin levels decreased and cognition improved in patients with AUD after 6 months of abstinence. The implications of these results are discussed here.

Our results suggest an association between reelin plasma levels and cognition at early states of AUD, in the sense that reelin is able to explain the presence of cognitive impaiment in this group.. Whether this association may be causative or a consequence of AUD pathology is unresolved at present. Reelin appears to be a protective factor that maintains normal brain function (Levenson et al., 2008), and the alteration of reelin human gene is thought to be involved in the pathogenesis of some neuropsychiatric disorders (Ovadia and Shifman, 2011; Wang et al., 2014; Bufill et al., 2015; Fehér et al., 2015; Li et al., 2015). In this study, higher plasma levels of reelin were observed in patients with AUD compared with controls. Altered reelin levels in the brain, CSF, or plasma have been reported in animal models and humans with several neuropsychiatric disorders (Ishii et al., 2016; Sánchez-Hidalgo et al., 2022). However, most studies are genetic or focused on brain reelin levels, and the exact contribution of peripheral reelin to cognition is largely unknown. Regarding plasma reelin, Fatemi et al. (2002) showed reductions in autistic patients, while another study found that the 50% of the children with autism showed no changes in reelin levels and the other 50% displayed a 30-fold increases, compared with controls (Cuchillo-Ibáñez et al., 2020).

The higher plasma reelin levels in the AUD group can be discussed based on several aspects. Firstly, it could be argued that reelin may be related to liver damage, as some studies indicate its involvement in liver fibrosis (Kobold et al., 2002; Carotti et al., 2017; Sturm et al., 2021) and suggest a protective effect in the liver (Botella-Lopez et al. 2008). However, we excluded patients with AUD with chronic hepatic damage and liver cirrhosis, making it unlikely that the high levels of reelin found in the AUD group were induced by a significant liver disease. Moreover, reelin plasma levels did not correlate with biomarkers of liver status, which were within normal ranges in the AUD group. Alternatively, alterations in reelin could be reflecting medication effects, as some studies have reported altered reelin levels in patients treated with antipsychotic or antidepressant treatments (Fatemi et al., 2009; Yin et al., 2020). In this study, disulfiram was the most frequently used medication (83.3%) in the AUD group, followed by antidepressants (45.8%), so we cannot rule out the potential effects of these medications; this limitation should therefore be considered. Additionally, it should be noted that we found an association between reelin and the length of alcohol abuse since last relapse so that the longer the time of dependent drinking, the higher the reelin levels. Thus, the elevations in reelin could be indicative of the course of illness because at 6 months of abstinence, when the disorder was in recovery, we found these levels normalized or even below controls.

Interestingly, patients with AUD showing cognitive deficits and the highest reelin levels were those who expressed APOE4 in plasma. The ApoE-ε4 allele is one of the genetic risk factors recently found to modulate cognition in alcohol consumption (Kim et al., 2012; Downer et al., 2014; Slayday et al., 2021). Most of the studies have focused on genetic variants, and very few studies have studied the presence of APOE4 protein in plasma. Determining plasma APOE4 not only identifies individuals carrying the allele ε4 of APOE (a genetic component indicated by a "yes/no" question) but also provides information about the quantitative levels of APOE4 protein reached in plasma. In this regard, epigenetic mechanisms and pathologies may influence the expression of the protein.

In our study we found an elevated percentage of APOE4+ among control patients. This was surprising in a random sampling of normal adults, according to the published prevalence in Europe (see supplementary Discussion 3.1). Anyhow, we did not observe differences in the levels of APOE4 reached in plasma by ε4 carriers in the control and AUD groups, and this constitutes an important data itself, suggesting that alcohol abuse did not interfere with plasma APOE4 expression. Interestingly, patients expressing plasma APOE4 had worse cognitive performance than no carriers in the AUD group but APOE4 presence in controls has no negative effect on cognition. The absence of cognitive impact of Apoe-ε4 in the control group was not surprising considering previous research showing an effect of Apoe-ε4 genotype in cognition in alcohol consumers but no effect in nondrinkers (Slayday et al., 2021).

While it would be reasonable to hypothesize that higher levels of APOE4 protein among Apoe-ε4 carriers would have a greater impact on cognition, we did not find an association between the quantity of plasma APOE4 in ε4-carriers in either the AUD group or the control group. However, it was the presence of plasma APOE4 (which identifies the genotype Apoe-ε4 carriers from noncarriers) that showed a relationship with worse cognition (memory/learning) in the AUD group. These results suggest that

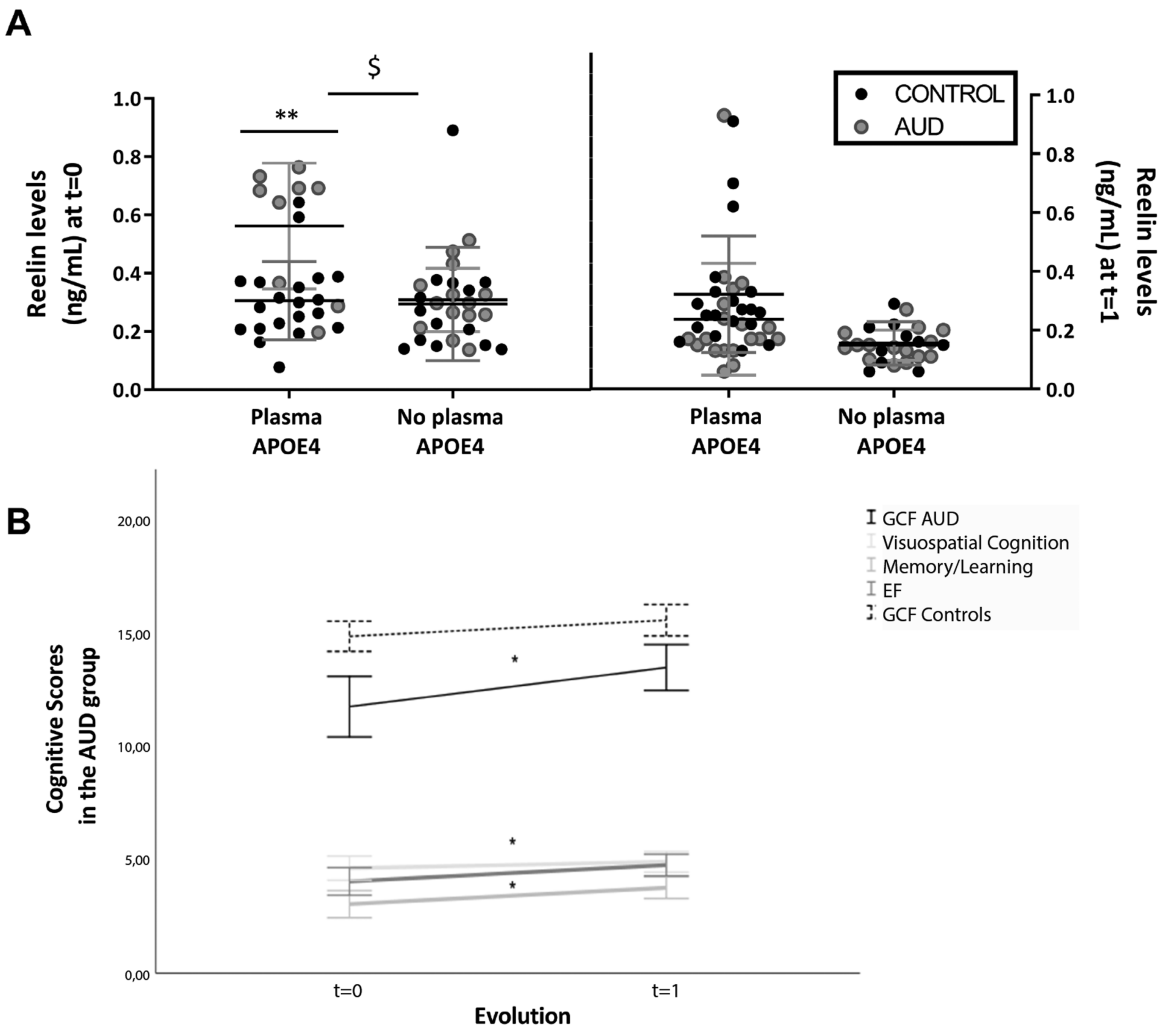

Figure 5. Evolution of reelin and cognition in the AUD group after 6 months of abstinence(t=1). (A) Reelin plasma levels in the AUD and control groups at t=0 (left panel) and t=1 (right panel) in patients expressing or not plasma APOE4. Two-way ANCOVAs, controlling for age and education. The statistical analyses in A (t=0) showed an overall effect of group $P<.01$; APOE4 $P<.05$ and analysis of the interaction (group*APOE4) $P<.05$. In A we represent the interaction with "$." Bonferroni post hoc test after interaction. (B) Cognitive evolution (GCF) in the control group (discontinued line) and in the AUD group (nondiscontinued lines) from t=0 to t=1. Two-way ANCOVA with group and APOE4 as factors, controlling for age and education. Effect of group*time *$P<.05$. Results are presented as mean (SD) in A and B. EF, executive function; GCF, general cognitive functioning.

the central APOE4 levels may play a fundamental role in AUD cognition rather than plasma levels. In contrast, reelin plasma levels served as good indicators of cognitive impairment during early abstinence. In our study, we found an inverse association between plasma reelin levels and cognition, with levels of reelin significantly elevated in Apoe-ε4 carriers within the AUD group. At 6 months of abstinence, as reelin levels decreased in the AUD group, we observed an improvement in cognitive function.

Our fundamental question therefore would be discerning the role of peripheral APOE4 and reelin in AUD-associated cognitive decline. The function of both proteins outside the CNS is currently poorly understood. Most of the studies focused on genetic variants or central levels of these proteins, and the specific contribution of peripheral reelin and APOE4 to the pathogenesis of neuropsychiatric disorders and associated cognitive impairment, as well as any potential abnormalities in the reelin/APOE4 pathway, remain largely unknown. New lines of evidence suggest a peripheral role of these proteins in the regulation of cognition. For example, peripheral APOE might have an impact in cerebrovascular and brain functions (Martínez-Morillo et al., 2014; Lane-Donovan et al., 2016). A very recent study, by developing mice models that express human APOE4 in the liver with no detectable levels in the brain, identified a role for peripheral APOE4 in synaptic plasticity and cognition, impairing cerebrovascular functions, independently of brain levels (Liu et al., 2022). Regarding reelin, it is primarily synthesized in the brain, and it has been suggested that the pools of plasma and CSF reelin have different origins (Botella-López et al., 2006). Circulating reelin appears to be produced largely by the liver (Smalheiser NR et al., 2000), and members of the reelin signaling pathway (e.g., VLDLR receptor) are expressed in the periphery, suggesting a role in the periphery.

The molecular mechanisms by which plasma reelin and APOE4 might contribute to AUD cognitive impairment are currently unknown. The known protective effects of reelin in synaptic plasticity and brain function (Martínez-Cerdeño et al., 2002; Weeber et al., 2002) and in the liver (Botella-Lopez et al., 2008) suggest that the elevations of reelin found in early abstinence in ε4 carriers may serve as a homeostatic signal to counteract alcohol-induced toxicity in the AUD group. In this regard, since reelin and APOE4 share receptors, it could be possible that circulating levels of APOE4 compete with reelin for binding to their shared receptors. In fact, in vitro studies have shown that the

interaction of reelin with lipoprotein receptors is inhibited when ApoE4 alleles are present (D'Arcangelo et al., 1999). Some studies showed that the isoform of APOE expressed by patients can differentially affect the reelin signaling. For instance, ApoE4 impaired the NMDA receptor phosphorylation by reelin, resulting in synaptic plasticity anomalies (Chen et al., 2010). The lack of reelin has been associated with hyperphosphorylation of Tau (D'Arcangelo et al., 1999; Hiesberger et al., 1999), leading to neuronal degeneration (cited by Botella-López et al., 2006), and reelin deficiency appears to be related to the onset of cognitive deficits in reelin-deficient mice (Falconer, 1951; Brigman et at., 2006; Krueger et al., 2006). Intriguingly, the APOE4 isoform impairs both receptors ApoER2 and VLDLR recycling to the surface upon reelin activation, leading to reelin resistance to signaling (Lane-Donovan and Herz, 2017). Hence, it is plausible to speculate that the organism may induce the release of reelin as a compensatory mechanism to overcome the signaling deficit in patients with AUD that are APOE4 carriers. However, this hypothesis requires thorough testing in future investigations.

Nevertheless, since ApoE-ε4 carriers in the control group did not exhibit alterations in reelin levels or cognition, some interactions driven by alcohol may have taken place. In this regard, alcohol exposure critically target elements in the ApoE/reelin signaling pathway in vitro followed by a sustained period during which the pathway itself can no longer be activated by application of reelin, suggesting that ethanol disrupts the reelin pathway in vitro (Wang et al., 2019; McClintick et al., 2020). Similar disruptions have also been described in vivo (Wang et al., 2021). Within the hippocampus, ethanol exposure leads to a reduction in reelin-positive interneurons, neuroinflammation, and suppression of neurogenesis (Takahashi et al., 2022). Both alcohol exposure (Jiang et al., 2021) and reelin deficiency (cited by Botella-López et al., 2006) have been implicated in tau protein phosphorylation in rodents, which is associated with neuroinflammation and neurodegeneration. Furthermore, it is well-known that alcohol abuse induces oxidative stress and lipid peroxidation, and it has been very recently hypothesized that these are important factors linked to derangements in the ApoE/Reelin-ApoER2 pathway (Ramsden et al., 2022). The release of reelin in the AUD group may be driven by an impaired signaling pathway in ApoE-ε4 carriers when there is alcohol on board, serving as a homeostatic signal to counteract this signaling deficit and explaining the elevated reelin levels during early abstinence. This hypothesis is speculative at the moment and it would need future confirmation.

Little information is available about the presence and function of VLDLR and ApoER2 in PBMCs, although both appear to be present in these immune cells (The Human Protein Atlas, https://www.proteinatlas.org/) (see supplementary Discussion 3.2).

We are aware of the limitations of this study. The small sample size influences the inclusion of covariates in the statistical analysis as only large differences appear significant in such cases. We controlled by age and education, but future studies will aim to control other important factors such as sex. Another aspect to be highlighted is that the novel e4Quant technique does not differentiate between homozygous and heterozygous APOE ε4/ε4, so we cannot compare our results with genetic studies. However, e4Quant is an efficient technique that discriminates APOE4 from other isoforms and, in the actual context of this study, the presence or absence of APOE4 in plasma (measured quantitatively) is a more valuable measure for us than genetic variants.

Both a limitation and a strength of this study is the young age group included. The influence of aging on cognition and expression of apolipoproteins has been documented (Muenchhoff et al. 2017), so studies involving middle-aged individuals are needed. In addition, the detrimental effects of alcohol appear to be more pronounced in the elderly than in young adults, particularly with regard to cognition (reviewed in Kim et al., 2012). Specifically, alcohol consumption in older adults has been associated with cognitive impairment in individuals who are APOE-ε4 carriers (Dufouil et al., 2000; Anttila et al., 2004), but the interaction between APOE-ε4 genotype and alcohol consumption on cognition has not been found in midlife adults (Downer et al., 2014). To date, very few studies have revised the risk of the presence of APOE4 in the relationship between alcohol consumption and cognition in middle-aged patients. Additionally, age might be a contributing factor to changes in brain reelin levels given declining reelin expression with age (Abraham and Meyer, 2003; Long et al.2020; Despotovski et al., 2021). In our study, the AUD group was older than the control group but still displayed the highest plasma reelin levels. These results suggest that the contribution of alcohol is greater than the potential influence of age.

Also, we do not have information regarding the levels of APOE4 and reelin in the brain of these patients, so we cannot draw direct conclusions about the effects of each biomolecule on brain function based on plasma levels alone. Brain and plasma reelin levels may have different cellular origins, and studies on plasma reelin are still scarce, so studies about the peripheral contribution of elements of the reelin/APOE4 pathway controlling for all confounded factors are encouraged.

In conclusion, our results show that reelin plasma levels peak in patients with AUD during early abstinence in those individuals who also express the APOE4 isoform in plasma, identifying worse cognitive performance. Control patients expressing APOE4 did not show any changes in reelin levels or cognition. Reelin levels correlated with the length of alcohol abuse in the past and levels normalized along evolution of the disorder together with a mild cognitive improvement, suggesting that reelin plasma levels could be a reflection of an homeostatic mechanism against toxic effects of alcohol in ε4 carriers.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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This study was approved by the Research Ethics Committee of the Hospital 12 de Octubre, Madrid (Spain), Nº CEIm: 19/002, and was conformed to the provisions of the World Medical Association Declaration of Helsinki. Each participant signed a written informed consent individually and all data were coded to maintain anonymity and confidentiality.

Interest Statement

The authors have no conflicts of interest to declare.

Data Availability

Patient personal information is confidential according to the law. Other data in the manuscript are freely available upon request to the corresponding author (lorio@psi.ucm.es).

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