Peripheral blood biomarkers coupled with the Apolipoprotein E4 genotype are strongly associated with semantic and episodic memory impairments in elderly subjects with amnestic mild cognitive impairment and Alzheimer's dementia.

Short title: Biomarkers of neurocognition

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Abstract

Background: The Apolipoprotein E4 (ApoE4) genotype is strongly associated with Alzheimer's disease (AD), although the presence of the ApoE4 allele alone is not sufficient to explain AD. The pathophysiology of amnestic mild cognitive impairment (aMCI) remains unclear. This study aims to examine associations between peripheral blood biomarkers coupled with ApoE4 and episodic and semantic memory.

Methods: The CERAD battery was completed and various biomarkers were assayed in 60 subjects with aMCI, 60 with AD and 62 healthy controls.

Results: Deficits in semantic and episodic memory were significantly predicted by anion gap and bicarbonate, albumin and glucose coupled with Apo E4. Furthermore, these peripheral biomarkers interacted with ApoE to predict greater memory impairments.

Conclusions: Peripheral blood biomarkers may interact with pathways related to ApoE4 to predict greater semantic and episodic memory impairments, thus contributing to the pathophysiology of aMCI and AD. Our data suggest that the transition from aMCI to AD could at least in some cases be associated with significant interactions between ApoE4 and those peripheral blood biomarkers.

Highlights

- Common peripheral blood biomarkers, including anion gap, bicarbonate, albumin and glucose coupled with ApoE4 had a significant effect on cognitive functions
- Those peripheral blood biomarkers may interact with the pathways associated with ApoE4 to predict greater memory impairments as compared to ApoE4 alone
- The transition from aMCI to AD may, in some cases, be explained by significant interactions between ApoE4 and those peripheral blood biomarkers.

Key words: episodic memory, apolipoprotein, dementia, biomarkers, anion gap, inflammation

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative and neuroinflammatory brain disorder, (1, 2) which is characterized by a gradual decline in various cognitive domains including learning and memory, attention, executive functions, language, perceptual-motor function and social cognition leading to meaningful impairments in functional status. (3) Moreover, AD patients experience difficulties in carrying out activities of daily living (ADL) and upholding social interactions, while a substantial subset of patients may develop behavioral and psychological symptoms. (4, 5) This progressive neurocognitive deterioration appears to be associated with the accumulation of amyloid plaques and tau protein in the brain, which are well known neurobiological correlates of AD. (6, 7)

The apolipoprotein epsilon 4 (ApoE4) allele is the most widely replicated genetic risk factor for AD. (8-10) Genome-wide association studies (GWASs) revealed that up to 40% of AD patients carry the ApoE4 allele, and that the risk for AD further increases among carriers of the E4/E4 genotype (odds ratio = 14.9). (11) In Thai patients with AD, ApoE4 carriers show lower scores on both the Thai version of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD-Th) and the Thai version of the short Boston Naming Test (T-BNT), (12, 13) while ApoE3 carriers exhibit better performance on ADL and social skills. (12) Nevertheless, the ApoE genotype in isolation is insufficient to explain all cases of AD, thus suggesting that interactions with other biomarkers could play a role in the pathophysiology of this neurodegenerative disorder.

Amnestic mild cognitive impairment (aMCI) is characterized by milder neurocognitive impairments including poor learning ability, deficits in episodic and semantic memory, as well as language difficulties, but without an associated decline in ADL. (14-18) Conversion rates to AD is greater in people with aMCI (14%) compared to the the general population (1-2%). (14) The underlying pathophysiology of aMCI remains unclear and an association between aMCI and ApoE4 has not been replicated across studies. (19) Furthermore, the risk of progression from aMCI to AD seems to occur independently of the ApoE genotype and amyloid neuropathology. (20)

However, recent findings suggest that not only brain processes, but also peripheral immune-inflammatory responses may play a role in the pathophysiology of AD. (21-23) For example, an alternative neurobiological model of AD proposes that the ApoE4 allele, along with an altered microRNA expression of *TREM2* (triggering receptor expressed on myeloid cells-2) and an increased transcription of *TNFA* (tumor necrosis factor alpha), could underpin peripheral and central immune-inflammatory responses implicated in the pathophysiology of AD. (23, 24) There is now evidence that peripheral blood pro-inflammatory biomarkers are increased in AD, including pro-inflammatory cytokines, homocysteine, C-reactive protein, chemokines, epidermal growth factor, adhesion molecules, and α1-antichymotrypsin. (22, 25, 26) Moreover, significant associations between impairments in neuropsychiatric test results and peripheral blood biomarkers in patients with aMCI and AD have been reported. (13, 27) For example, hypercholesterolemia, low serum high-density lipoprotein (HDL) and high serum low-density lipoprotein, low serum B12, low homocysteine levels, altered serum metabolomics as well as a decreased level of red blood cell folate, are thought to promote neurotoxicity and cognitive dysfunction seen in patients with aMCI and AD. (27-30) Other promising peripheral blood biomarkers that are associated with

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neurocognitive impairments in AD are glucose, (31-33) and anion GAP (AGAP) and bicarbonate (HCO3-) levels. (34) Furthermore, impairments in naming as measured with the T-BNT were significantly associated with ApoE4 coupled with lower folic acid levels along with an increased plasma triglyceride / HDL-cholesterol ratio, i.e. the atherogenic index of plasma (AIP). (13) We reported that ApoE4 significantly predicted impairments in episodic and semantic memory in patients with aMCI and AD. (12) However, there are no data to indicate whether peripheral biomarkers such as glucose, AGAP, bicarbonate, folic acid, AIP, as well as lower albumin (inflammatory biomarkers) could interact with ApoE4 to enhance the prediction of episodic and semantic memory impairments in elderly with aMCI and AD.

Thus, this study aims to determine whether peripheral blood biomarkers and ApoE4 have cumulative or moderator effects on CERAD test scores including verbal fluency test (VFT), world list memory (WLM), WL Recall and WL Recognition tests. The specific hypothesis was that a combination of ApoE4 and some of those biomarkers could enhance the prediction of memory deficits in aMCI and AD.

Subjects and Methods

Subjects

Data for this study were drawn from a cross sectional database registry study for dementia funded by the Thai Government, which recruited 182 Thai participants of both sexes and ages ranging from 55 to 90 years. (12, 35) Participants with memory impairment were enrolled from the Dementia clinic, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. Healthy control participants were recruited from senior Red Cross volunteers at King Chulalongkorn Memorial Hospital and community healthy aging senior club members, in Pathumwan District area, Bangkok, Thailand. Patients and HCs were recruited from the same catchment area (Pathumwan district, Bangkok province). All participants were allocated in three groups, namely 60 with amnestic mild cognitive impairment (aMCI), 60 with Alzheimer's disease (AD) and 62 normal control (NC).

aMCI was diagnosed according to Petersen's Criteria. (14) Additional inclusion criteria were: (a) MMSE-Thai score higher than 23 and (b) a CDR score of 0.5. A diagnosis of AD was made using criteria of the National Institute of Neurological and Communication Disorders and Stroke/AD and Related Disorders association (NINCDS-ADRDA). (36) Additional inclusion criteria for AD were: (a) Thai version of the Mini-Mental State Examination 2002 (MMSE-Thai) (37) score between 10 and 23; (b) Thai version of Clinical Dementia Rating Scale (CDR) score between 1 and 2; and (c) impaired ADL, as assessed using the Blessed Dementia Scale for ADL. Controls did not report any subjective memory complaints and had a CDR score of 0 and a MMSE score more than 23. Exclusion criteria for patients and HCs were: (a) neurologic diseases: Parkinson's disease, multiple sclerosis, meningitis, encephalitis, epilepsy, traumatic brain injury; (b) medical diseases including severe heart disease (functional class II or more), chronic obstructive pulmonary disease (exacerbation more than 3 times/month), uncontrolled hypothyroidism, vitamin B12 deficiency, chronic kidney disease and cancer; (c) other dementia syndromes, including vascular dementia and frontotemporal lobe dementia, (d) axis 1 psychiatric disorders, including schizophrenia, substance abuse and major depressive disorder according to DSM-IV-TR criteria and a Thai Geriatric

Depression Scale (TGDS) > 12; and (e) abnormal laboratory tests such as thyroid function tests, vitamin B12 levels, VDRL and anti-HIV.

All participants and all guardians of patients with MCI and AD provided written informed consent prior to participation in this study. The study was conducted according to Thai and international ethics and privacy laws. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (No 359/56), which is in compliance with the International Guideline for Human Research protection as required by the Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Methods

A senior psychiatrist and neurologist with expertise in dementia assessed each participant based on clinical history, physical and neurological examination and the Clinical Dementia Rating (CDR). (38) A registered clinical psychologist who was blinded from the clinical diagnosis and CDR scores assessed all participants using the CERAD-NP. We *a priori* selected 4 out of 8 neuropsychological subtests from the CERAD-NP namely the Word List Memory (WLM) to probe verbal episodic memory or immediate working memory, Word List Recall (WLRecall) to probe the ability to recall (delayed recall) and verbal episodic memory recall, and Word List Recognition (WLRecognition) to probe verbal learning recall recognition or verbal episodic memory-discriminability. (39)

MRI brain scans and blood sampling were performed following a protocol for all participants in the CERAD-Th project including 1.5T MRI scans, basic blood tests, biochemical parameters, and biomarkers including complete blood count, fasting blood sugar, serum electrolytes, liver function test (ALT, AST), lipid profile (total cholesterol, high density lipoprotein, low density lipoprotein), serum uric acid, kidney function test (blood urea nitrogen, creatinine kinase and glomerular function rate), thyroid function test (free T3 and T4, TSH), serum B12 level, serum folate, serum calcium and apolipoprotein phenotype. All blood assays were performed in the Central Laboratory at the King Chulalongkorn Memorial Hospital, which is accredited conforming to ISO 15189 standards.

Laboratory Assays

Fasting blood was collected for all participants between 8.00 and 8.30 a.m. All biochemical parameters mentioned above were assayed by the Central Laboratory, Department of Laboratory Medicine, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. This study used 3 mL clotted blood (serum), centrifuged at 1,000 g during 5 minutes and measured using Architect C8000 (Abbott Laboratories, Abbott Park, Illinois, USA) for the following parameters. Plasma glucose was measured by Hexakinase/ G-6-PDH technique with inter-assay coefficients of variability (CV) of 2.0%. Blood electrolytes (Sodium, Potassium and Chloride) were measured by indirect ion selective electrode (CV 1.3%, 1.4% and 1.2%, respectively). Blood carbon dioxide was measured by PEP carboxylase method (CV 5.9%). Total cholesterol was measured by enzymatic assay (CV 1.6%). Triglyceride was measured by glycerol phosphate oxidase (CV 1.8%). HDL-cholesterol was

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measured by accelerator selective detergent (CV 4.0%), and LDL-cholesterol was measured by liquid selective detergent (CV 7.4%). Folate levels were measured using electrochemiluminescence immunoassay (ECLIA) as analyzed by the Cobas 6.000 Analyzer (Roche, Germany). The inter-assay CV values are < 9.8%.

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures with a DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). DNA was amplified by using two primers, 50-ACAGAATTCGCCCCGGCCTGGTACACAC-30 and 50-TAAGCTTGGCACGGCTGA AGGA-30. Each amplification reaction contained 1:g ofleukocyte DNA, 1 pmol/:l of each primer, 10% dimethyl sulfoxide, and 0.025 units/pl of Taq polymerase in a final volume of 30 :l. Each reaction mixture was heated at 95C for 5 min followed by 40 cycles of 95 _C for 60 s, 65 _C for 80 s and 72 _C for 80 s with a final extension at 72 _C for 7 min. The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, USA) according to the protocols supplied by the manufacturer, and sent for direct sequencing to Macrogen Inc., Seoul, South Korea. In the current study, we used the ApoE4 genotype group (any E4 allele) comprising participants carrying E4/E4 (n=6), E3/E4 (n=32) and E2/E4 (n=5). Indeed, one E4 copy (especially E3/E4) increases risk for AD, while two E4 copies (E4/E4) increase risk considerably. (40, 41)

Statistics

Analysis of variance (ANOVA) was employed to assess differences in scale variables among study groups (divided according to the median-split method applied on the total CERAD score). Analysis of contingence tables (X^2-tests) was used to assess associations between categorical variables among the study groups. Correlations between scale variables were computed using Pearson's product moment correlation coefficients or Spearman's rank order correlation coefficients. Hierarchical binary logistic regression analysis was used to assess the significant explanatory variables (biomarkers, age, sex and education) which predict the study sample with lowered total CERAD scores. Odds ratios and upper-lower 95% confidence intervals were computed and we employed Nagelkerke values as an estimate of the effect size. Hierarchical multiple regression analysis was employed to delineate the most significant predictors (biomarkers combined with age, sex and education) of the total CERAD score and VFT, WLM, WLRecall and WLRecognition scores. All regression analyses were checked for multicollinearity. The results of the statistical analyses were bootstrapped (1000 bootstraps) and bootstrapped results are reported in case they show a different outcome. Principal component analysis (PCA) was used to extract the first PC from memory tests and the PC score was used in subsequent analyses. Tests were two-tailed and a p-value of 0.05 was used for statistical significance.

We used multilayer perceptron (MLP) Neural Network (NN) analyses (SPSS 25) to investigate the complex (non-linear) associations between the CERAD tests scores (output variables) and peripheral blood biomarkers, APOE4, age, sex and education (input variables) in automated feedforward architecture models. We trained the network employing one or two hidden layers with a variable number of nodes (up to 10) and used batch and minibatch (gradient descent with 10-30 epochs) types of training. We assigned 46.67% of the cases to the training set (in order to

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estimate the network parameters), 20.0% to the testing set (to prevent overtraining) and 33.33% to the holdout set (to evaluate the final network). As stopping rule, we used one consecutive step with no further decrease in the error term. We also computed error, relative error and importance of each of the input variables in sensitivity analyses. All statistical analyses were performed using IBM SPSS windows version 25.

Results

Socio-demographic and clinical data

Table 1 shows the socio-demographic data in both subjects with lower versus higher CERAD scores (divided according to the median split method). Subjects with lower CERAD scores had higher age, less education, and lower scores on the total CERAD, VFT, WLM, WL Recall and WL recognition than those with a CERAD score > 73. Subjects with a CERAD score < 73 had higher glucose, WBC numbers, AGAP, and AIP index than those with a higher CERAD score, while HCO3- and folic acid were significantly lowered.

Inspection of the intercorrelation matrices shows that age is associated with CERAD subdomain scores and with most biomarker data, namely VFT (r=-0.462, p<0.001, n=182), WLM (r=-0.520, p<0.001, n=182), WLRecall (r=-0.511, p<0.001, n=182) and WLrecognition (r=--0.406, p<0.001, n=182), glucose (r=194, p=0.009, n=182), albumin (r=0.359, p<0.001, n=181), HCO3- (r=-0.159, p=0.033, n=180), AIP (r=0.186, p=0.015, n=172), folic acid (r=-0.269, p<0.001, n=182) and WBC count (r=0.207, p=0.005, n=182), but not AGAP (r=0.123, p=0.101, n=179).

Results of logistic regression analysis

Table 2 shows the results of logistic regression analysis with the group with a CERAD score <73 as dependent variables (and those with higher levels as reference group). The first regression shows that education (inversely) and age, any E4 and AGAP significantly predict membership to the lower CERAD group (χ^2=89.76, df=4, p<0.001, Nagelkerke=0.557); 83.1% of all cases were correctly classified with a sensitivity of 79.3% and a specificity of 86.9%. The second regression shows that education and HCO3- (both inversely) and any E4 and age (both positively) predict membership to the lower CERAD group (χ^2=93.65, df=4, p<0.001, Nagelkerke=0.562); 81.3% of all cases were correctly classified with a sensitivity of 78.2% and a specificity of 84.5%.

Predicting neurocognitive test results in the whole sample

Table 3 shows the prediction of the total CERAD score and the different subdomains using the biomarkers in addition to ApoE4, age, education and sex. Firstly (results shown in table 3), we will describe the results of multiple regression analyses with the CERAD tests as dependent variables and AopE4 and the biomarkers as primary explanatory variables, while adjusting for sex and education. Age was not entered as a covariate in order to allow age-dependent biomarkers (including glucose, folic acid, AIP, glucose) to predict the cognitive test results without interference with age. Subsequently, we will describe the results of a second regression analysis with age as an additional covariate.

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Table 1. Demographic and clinical data of patients with a low total CERAD (<73) versus a higher CERAD score.

Variables	CERAD Total>73 N=89	CERAD Total<73 N=93	F/X^2	df	p
Age	69.5 (6.2)	77.9 (6.8)	74.19	1/180	<0.001
Sex (male/female)	20/69	26/67	0.72	1	0.395
Education	12.5 (4.9)	7.2 (5.5)	47.05	1/180	<0.001
CERAD Total	83.9 (5.7)	46.7 (16.5)	405.94	1/180	<0.001
VFT	21.0 (3.0)	11.2 (4.7)	274.60	1/180	<0.001
WLM	22.5 (3.0)	11.7 (5.6)	261.98	1/180	<0.001
WL True Recall	8.4 (1.4)	2.7 (2.8)	297.54	1/180	<0.001
WL Recognition	9.6 (0.9)	5.1 (3.4)	147.68	1/180	<0.001
FBG (mg/dL)	97.4 (18.7)	105.4 (22.0)	6.95	1/179	0.009
HCO_3^- (mmol/L)	27.34 (2.59)	26.27 (3.10)	6.13	1/178	0.014
AGAP (mEq/L)	12.55 (2.28)	13.62 (3.05)	7.14	1/177	0.008
WBC ($x10^9$/L)	5.88 (1.40)	6.51 (1.69)	7.59	1/180	0.006
Vitamin B9 (ng/dL)	15.26 (4.49)	13.09 (4.86)	9.88	1/180	0.002
AIP (mg/dL)	2.07 (1.63)	2.54 (1.68)	3.53	1/170	0.062
Albumin (g/dL)	4.28 (0.24)	4.18 (0.27)	6.60	1/179	0.011

CERAD: The Consortium to Establish a Registry for Alzheimer's Disease
VFT: Verbal Fluency Test
WLM: Word List Memory
FBG: Fasting blood glucose
HCO3-: Bicarbonate
AGAP: Anion GAP
WBC: White blood cells
AIP: Atherogenic index of plasma

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Table 2 Results of binary logistic regression analysis with the study sample characterized by a low total CERAD score (<73) as dependent variable.

Experimental Variables	BE	SF	W	df	p	OR	95% CI
Education	-1.05	0.24	19.13	1	<0.001	0.35	0.22 – 0.56
Age	1.32	0.26	25.15	1	<0.001	3.75	2.24 – 6.28
Any E4	1.50	0.59	6.52	1	0.011	4.48	1.42 – 14.15
AGAP	0.51	0.22	5.67	1	0.017	1.67	1.10 – 6.28
Education	-1.11	0.24	21.16	1	<0.001	0.33	0.21 – 0.53
Any E4	1.55	0.57	7.33	1	0.007	4.71	1.53 – 14.43
HCO3-	-0.43	0.21	4.16	1	0.041	0.65	0.43 – 0.98
Age	1.29	0.26	24.36	1	<0.001	3.65	2.18 – 6.10

AGAP: Anion gap

HCO3-: bicarbonate

OR: Odd's ratio, 95% CI: 95% confidence intervals with lower and upper limits

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Table 3 Results of multiple regression analyses with the total CERAD score, the Verbal Fluency Test (VFT) score, Wordlist memory (WLM) score and scores on the WL Recall and WL Recognition as dependent variables.

Dependent Variables	Explanatory Variables	B	SE	t	P	F model	df	p	Partial Eta squared
CERAD Total	Education	0.422	0.065	6.49	<0.001	19.64	6/158	<0.001	0.427
	Any E4	-0.651	0.148	-4.41	<0.001				
	Glucose	-0.135	0.065	-2.09	0.038				
	Albumin	-0.148	0.061	2.42	0.017				
	Sex	0.383	0.151	2.53	0.012				
	AGAP	-0.200	0.062	-3.20	0.002				
VFT	Education	0.373	0.069	5.42	<0.001	16.68	6/158	<0.001	0.388
	Glucose	-0.222	0.066	-3.37	0.001				
	Albumin	0.163	0.067	2.44	0.016				
	Any E4	-0.500	0.157	-3.19	0.002				
	HCO_3^-	0.209	0.067	3.13	0.002				
	Sex	0.397	0.160	2.49	0.014				
WLM	Education	0.372	0.069	5.42	<0.001	18.71	4/160	<0.001	0.319
	AGAP	-0.246	0.064	-3.87	<0.001				
	Sex	0.625	0.155	4.03	<0.001				
	E4	-0.626	0.159	-3.94	<0.001				
WL Recall	Education	0.366	0.066	5.55	<0.001	18.62	5/159	<0.001	0.369
	AGAP	-0.260	0.061	-4.28	<0.001				
	Any E4	-0.632	0.152	-4.17	<0.001				
	Sex	0.444	0.148	3.00	0.003				
	Albumin	0.159	0.062	2.55	0.012				
WL Recognition	Education	0.360	0.070	5.14	<0.001	15.26	4/160	<0.001	0.276
	Any E4	-0.645	0.164	-3.94	<0.001				
	HCO3-	0.224	0.066	3.36	0.001				
	Sex	0.368	0.159	2.31	0.022				

AGAP: anion gap

HCO3-: Bicarbonate

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The first regression in Table 3 shows that 42.7% of the variance in the total CERAD score was explained by the regression on ApoE4, glucose and AGAP (all inversely) and education and sex (positively). Introduction of age shows that glucose and albumin were no longer significant and that age, education, ApoE4, AGAP and sex significantly predicted 46.8% of the variance in the total CERAD score ($F=32.27$, $df=5/173$, $p<0.001$). AGAP remained highly significant ($t=-3.43$, $p=0.001$).

The second regression shows that 38.8% of the variance in VFT scores was explained by education, albumin, HCO3- and sex (all positively) and glucose and any E4 (inversely). Introduction of age shows that albumin was no longer significant and that 40.3% of the variance in VFT was explained by the regression on age, education, AopE4, sex, glucose and AGAP ($F=19.34$, $df=6/172$, $p<0.001$) and that HCO3- ($t=2.79$, $p=0.006$) and glucose ($t=-2.69$, $p=0.008$) remained significant.

We found that 31.9% of the variance in the WLM score was predicted by education and sex (both positively) and AGAP and ApoE4 (both negatively). Entry of age shows that age, education, ApoE4, sex and AGAP together explain 42.0% of the variance in WLM and that the effect of AGAP remained significant ($t=-3.22$, $p=0.002$).

Up to 36.9% of the variance in WLRecall was explained by the regression on education, sex and albumin (all positively) and AGAP and ApoE4 (both inversely). After introducing the effects of age, we found that age, education, ApoE4, sex and AGAP explained a large part of the variance in WLRecall (43.2%, $F=26.34$, $df=5/173$, $p<0.001$) and that AGAP remained significant ($t=-3.68$, $p<0.001$).

The last regression in table 3 shows that 27.6% of the variance in WLRecognition was explained by education, HCO3- and sex (all positively) and ApoE4 (inversely). After introducing age in this regression, we found that sex was no longer significant and that 30.9% of the variance in WLRecognition was explained by the regression on age, education, ApoE4 and HCO3- ($F=19.56$, $df=4/175$, $p<0.001$) and that HCO3- had a significant effect ($t=2.65$, $p=0.009$).

Are there any moderator effects of ApoE4?

Consequently, we have examined whether ApoE4 yields significant moderator effects in predicting the different CERAD tests. Towards this end we used univariate regression analyses to examine the interaction effects EpoE4 with the most important biomarkers established in table 3, namely AGAP (or HCO3-), glucose and albumin. We could not find any moderator effects of ApoE4 with these biomarkers on total CERAD score and VFT. The first regression in **Table 4** shows that an interaction between ApoE4 and glucose was a significant predictor of WLM showing that, in the presence of ApoE4, glucose has a significant negative effect on WLM. The second regression in Table 4 shows that WLRecall is significantly predicted by the interaction between no-APOE4 X albumin, indicating that when ApoE4 is not present, lowered levels of albumin are associated with lower WLRecall. The last regression in Table 4 shows that an interaction between AopE4 X AGAP significantly predicted WLRecognition, indicating that in the presence of ApoE4, AGAP has a significant negative effect on WLRecognition.

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Table 4. Results of univariate GLM analysis with Wordlist memory (WLM) score and scores on the WL Recall and WL Recognition as dependent variables.

Dependent Variables	Explanatory Variables	B	SE	t	p	F model	df	p	Partial Eta squared
WLM	Age	-0.331	0.064	-5.14	<0.001	17.99	7/165	<0.001	0.433
	Education	0.248	0.065	3.80	<0.001				
	Any E4	0.490	0.143	3.42	0.001				
	Sex	-0.435	0.152	-2.86	0.005				
	Glucose	-0.11	0.072	-0.15	0.877				
	E4 * glucose	-0.306	0.142	-2.15	0.033				
	AGAP	-0.179	0.062	-2.87	0.005				
WL Recall	Age	-0.293	0.067	-4.39	<0.001	18.51	7/195	<0.001	0.440
	Education	0.288	0.065	4.46	<0.001				
	Any E4	-0.553	0.141	-3.91	<0.001				
	Sex	-0.300	0.144	-2.08	0.039				
	Albumin	0.142	0.071	1.99	0.048				
	NoE4 * Albumin	0.324	0.139	2.33	0.021				
	AGAP	-0.212	0.059	-3.59	<0.001				
WL Recogntion	Age	-0.261	0.067	-3.91	<0.001	17.90	5/169	<0.001	0.346
	Education	0.219	0.065	3.35	0.001				
	Any E4	-0.608	0.150	-4.05	<0.001				
	AGAP	-0.085	0.072	-1.18	0.239				
	E4 * AGAP	-0.392	0.144	-2.72	0.007				

AGAP: anion GAP

E4: any ApoE4

Associations between neurocognitive tests in aMCI

In order to examine the associations between neurocognitive tests and biomarkers in aMCI we have carried out extra analyses in the subgroup of aMCI patients and controls. The first regression in **Table 5** shows that 41.2% of the variance in the total CERAD score was explained by age, education and AGAP. Also, 24.6% of the variance in VFT was predicted by age, education and glucose levels. In order to examine the association with episodic memory we have used the first principal component (PC) extracted from the three episodic memory tests (namely WLM, WLRecall and WLRecognition). This first PC explained 90.7% of the variance and all three test values loaded highly on this first PC (all > 0.938). Table 3 shows that 34.1% of the variance in this first PC extracted from the three memory tests was predicted by education (positively) and age, AGAP, glucose and the interaction ApoE4 X glucose. This interaction shows that when ApoE4 is present, glucose has a significant inverse effect on the memory latent vector.

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Table 5. Results of multiple regression analysis performed in the study groups of amnestic mild cognitive impairment and healthy controls with the total CERAD score, the Verbal Fluency Test (VFT) score and the first principal component extracted from the memory test scores (PC Memory) as dependent variables.

Dependent Variables	Explanatory Variables	B	SE	t	P	F model	df	p	Partial Eta squared
Total CERAD	Age	-0.189	0.034	-5.53	<0.001	27.28	3/117	<0.001	0.412
	Education	0.205	0.034	6.02	<0.001				
	AGAP	0.098	0.0393	-2.53	0.013				
VFT	Age	-0.261	0.067	-3.91	<0.001	12.59	3/116	<0.001	0.246
	Education	0.219	0.065	3.35	0.001				
	Glucose	-0.143	0.060	-2.37	0.019				
PC Memory	Age	-0.354	0.078	-4.54	<0.001	9.75	6/113	<0.001	0.341
	Education	0.251	0.077	3.25	0.003				
	AGAP	-0.273	0.088	-3.09	0.004				
	Glucose	-0.013	0.090	0.14	0.795				
	Any E4	-0.312	0.232	-1.34	0.030				
	E4*Glucose	-0.812	0.302	-2.69	0.014				

AGAP: anion gap.

E4 * Glucose: interaction between ApoE4 and glucose levels

PC memory: first principal component extracted from Word List Memory, Word List True Recall and Word List Recognition

Results of neural networks

Neural Network models were used to predict the total CERAD score (model 1), VFT score (model 2) and the first PC extracted from the 3 episodic memory tests (model 3) (output variables) using all biomarkers (including any ApoE4) and age, sex and education as input variables. Automatic architecture training of the network delineated the best models. **Table 6** shows the model summary of the trained Neural Networks. The three models were trained with 1-2 hidden layers with a variable number units in layer 1 (5-6) and layer 2 (4-5). The first layer used hyperbolic tangent as activation function and the output layer either identity or hyperbolic tangent as activation factor. In the three models, the sum of squares error term was minimized during the training, indicating that the three models learnt to generalize from the trend. The relative error terms in the training, testing and holdout samples were fairly constant, suggesting that the models are not overtrained (overfitted). The Spearman correlation coefficient between the CERAD, VFT and memory test scores and the model-predicted values are shown in Table 6 for the three models.

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Table 6. Results of neural networks showing network information, the model summary and the correlation coefficient between the CERAD test score and the model predicted values.

Network information	Layers / errors	Model 1: CERAD	Model 2: VFT	Model 3: Episodic memory PC
Number units	-	13	13	13
Hidden layers	Number hidden layers	2	2	2
	Number units in layer 1	6	5	5
	Number units in layer 2	5	4	4
	Activation function	Hyperbolic tangent	Hyperbolic tangent	Hyperbolic tangent
Output layer	Activation function	Identity	Identity	Hyperbolic tangent
Model summaries				
Training sample	Sum of squares error	19.123	18.724	6.763
	Relative error	0.425	0.499	0.525
Testing sample	Sum of squares error	5.418	7.895	3.341
	Relative error	0.361	0.545	0.516
Hold-out sample	Relative error	0.589	0.609	0.776
Correlations	With predicted value	0.739 (p<0.001)	0.679 (p<0.001)	0.641 (<0.001)

Model 1, 2 and 3: output variables were the total CERAD score, verbal fluence test (VFT) score and the first principal component extracted from the memory test scores (Word List Memory, Word List True Recall and Word List Recognition), respectively. All biomarkers, age, sex and education were entered as input variable

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Figures 1, 2 and 3 show the relevance chart displaying the importance and normalized importances of the 11 input variables. Figure 1 shows that education and age were the most important determinants of the predictive power of model 1 (prediction of total CERAD score), followed at a distance by albumin, AGAP, Any E4 and glucose. Figure 2 shows that education and age were the most important determinants of the predictive power of model 2 (prediction of the VFT score), followed at a distance by Any E4, HCO3-, and again at a distance by AIP, glucose and folic acid. Figure 3 shows that education, Any E4, AGAP and age were the most important determinants of the predictive power of model 3 (prediction of the memory PC score), followed at a distance by albumin and glucose.

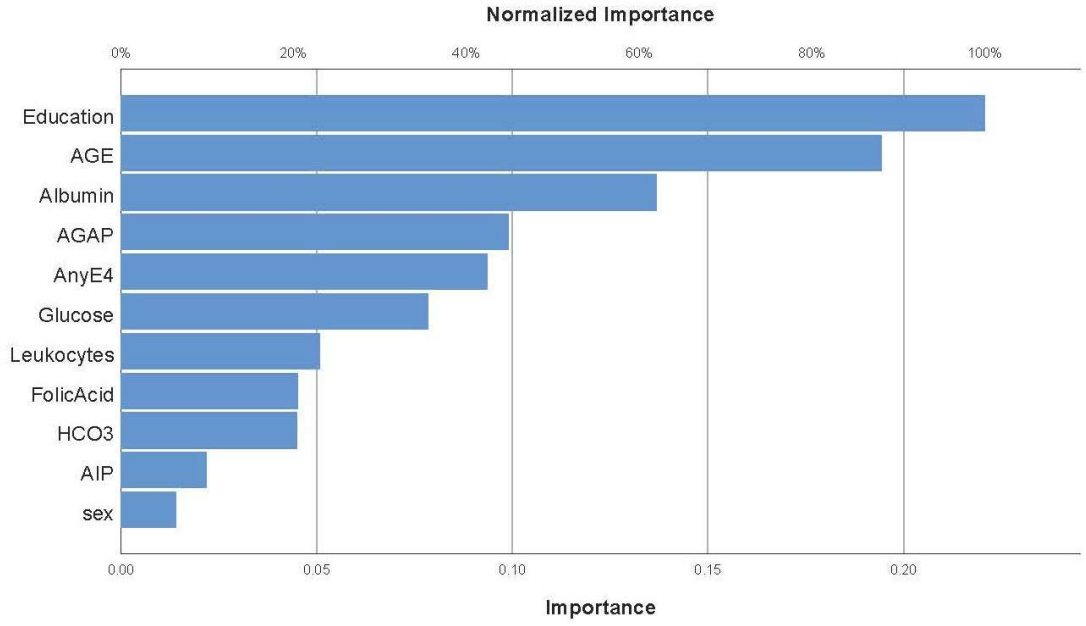

Figure 1. Results of neural network (relevance chart) with the total CERAD score as output variable. Education and age were the most important determinants of the predictive power of this model (prediction of total CERAD score), followed at a distance by albumin, anion GAP (AGAP), any E4 and glucose. Number of leukocytes, folic acid levels, bicarbonate (HCO3), atherogenic index of plasma (AIP) and sex were less important.

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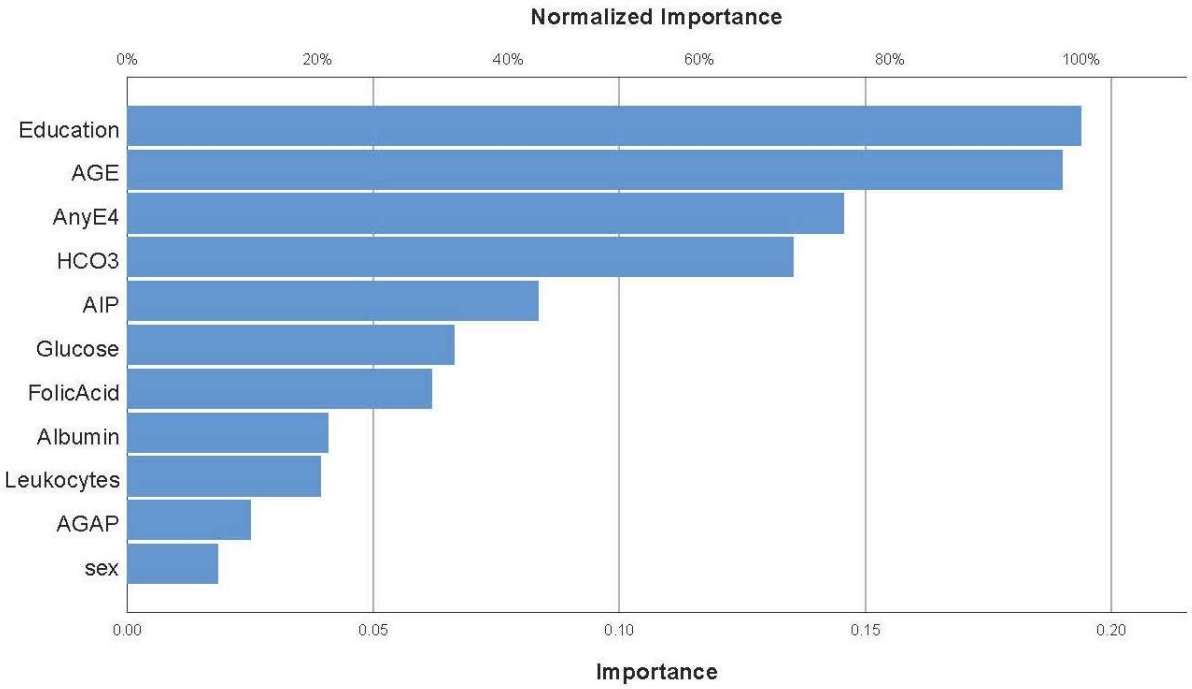

Figures 2. Results of neural network (relevance chart) with Verbal Fluency score as output variable. Education and age were the most important determinants of the predictive power of this model, followed at a distance by any E4 and bicarbonate (HCO3) and again at a distance by the atherogenic index of plasma (AIP), glucose and folic acid, while albumin, number of leukocytes, anion GAP (AGAP) and sex were not significant.

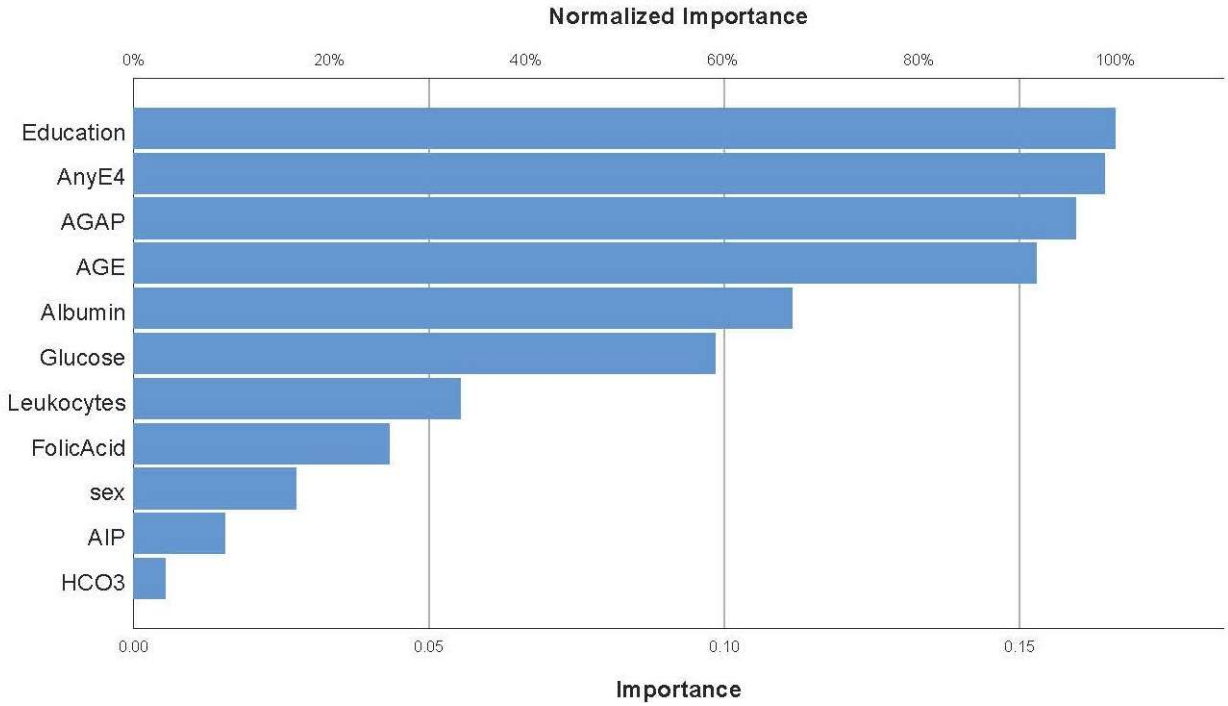

Figure 3. Results of neural network (relevance chart) with the first principal component extracted from three episodic memory tests as output variable.

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Education, any E4, anion GAP (AGAP) and age were the most important determinants of the predictive power of this model, followed at a distance by albumin and glucose. Number of leukocytes, folic acid, sex, the atherogenic index of plasma and bicorbonate (HCO3) were not important.

Discussion

The major finding of the study is that impairments in semantic and episodic memory (as measured with the CERAD-NP battery) are significantly predicted by blood biomarkers including anion gap and bicarbonate, albumin, glucose levels and Apo E4. There is now evidence that ApoE4 interferes with cognitive functions, including executive abilities and episodic memory. (42) For example, ApoE4 carriers and especially E4/E4 homozygotes show lowered levels on total CERAD score, VFT, WLM, WL recall and WL Reognition, indicating that ApoE4 has a strong impact on semantic and episodic memory scores. (12) During the normal ageing process, the presence of ApoE4 is associated with neurocognitive declines in episodic, working, and semantic memory. (43-46) Moreover, ApoE4 carriers show a higher risk to develop AD because ApoE4 promotes memory deficits through effects on brain neural networks. (47) The latter effects are probably related to increased cerebral amyloid angiopathy, lower clearance of amyloid-β (Aβ) peptide thereby causing Aβ excess, higher lipid levels, increased oxidative stress as well as a pro-inflammatory state. (48, 49) (50-52)

However, not all people who carry ApoE4 alleles and not all E4/E4 homozygotes develop AD, (53) indicating that ApoE4 may interact with other biomarkers or pathways to increase the risk for AD. In this respect, we found that combining ApoE4 with anion gap or bicarbonate levels, glucose or albumin levels (indicating peripheral inflammation) improved the prediction of CERAD, VFT and episodic memory scores. Our findings suggest that these peripheral biomarkers could interact with ApoE4-related pathways to promote greater deficits in semantic and episodic memory, and that those interaction enhanced the prediction of those cognitive deficits in our sample compared to ApoE4 alone.

Our results extend those of previous papers reporting that lowered bicarbonate levels may be associated with lower performance on cognitive tests including cognitive or executive impairments. (54, 55) For example, in a large cohort of individuals with hypertension, higher serum bicarbonate levels were associated with better global cognitive and executive functioning, but not with memory.(54) The latter authors computed that a decrease of 1 mEq/L in bicarbonate had the same impact on global cognitive functioning as being 4.3 months older. Lowered bicarbonate levels are also associated with confusion, cognitive impairment and delirium. (56) Recent preclinical data show that increasing bicarbonate concentrations stimulate the excitability of pyramidal neurons in the rodent hippocampus (57) providing further support for a putative role of acid-base homeostasis as a regulator of central nervous system activity. (57) While increases in pH may increase neuronal activity, a lowered pH may be accompanied by lowered neuronal activity and important mediators of synaptic function and memory including glutamate receptors appear to be pH-dependent. (58) It should be added that acidosis also accompanies other neurological disorders, including Parkinson's

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disease, multiple sclerosis and epilepsy. (59) An acidic environment may activate acid-sensing ion channels which may promote neuroinflammation, demyelination, axonal degeneration, endoplasmatic reticulum stress and oxidative pathways along with a diminishment of glutamate uptake. (59) Those mechanisms in conjunction provide further support to the findings herein reported. In this respect, our analysis not only showed cumulative effects of anion gap coupled with ApoE4 in predicting a decrease in cognitive functions, but also a specific interaction pattern between ApoE4 and anion gap in predicting lowered WL recognition test scores. In keeping with this view, among ApoE4 carriers, a higher anion gap was accompanied by lowered recognition test scores. These cumulative and interactive effects of ApoE4 and increased anion gap or lowered bicarbonate levels may be mechanistically explained by an increased impact on, for example, pro-inflammatory responses and oxidative stress pathways.

Previous research found lowered plasma albumin together with increased IL-6 levels in AD indicating a peripheral immune-inflammatory response in those patients. (60, 61) IL-6 plays a role in acute and chronic inflammatory responses and in the pathogenesis of neuroinflammatory disorders including AD. (62) There is also some evidence that lowered albumin levels are associated with cognitive impairments. For example, people with very low baseline albumin levels experience an accelerated decline in MMSE scores over a 13-year follow-up compared to those with higher albumin levels. (63) Glucose plays a pivotal role as an energy source to the brain by enhancing ATP biosynthesis (64) and many studies reported that glucose is associated with better outcomes on neurocognitive tasks. (65, 66) On the other hand, in elderly people, glucose dysregulation is associated with memory impairments, especially in episodic memory. (67, 68) Moreover, poorly controlled diabetes is accompanied by memory impairments and executive dysfunctions, (31) whilst in diabetic patients, increased glucose peaks may be a risk factor for cognitive decline and even dementia. (69) Other studies reported that blood glucose, insulin resistance and type 2 diabetes mellitus are major factors contributing to an increased risk of AD. (64, 70) Blood glucose crosses the blood-brain-barrier and may be detrimental to brain function through several mechanisms, including impaired radical scavenging and glutathione cycle dysfunctions, free radical generation, aberrant osmolality in brain cells and increased synthesis of advanced glycation end-products. (71) Moreover, glucose fluctuations induce glial toxicity with the generation of glutamatergic oxidative products and may also lead to inflammation, neurotoxicity and neuronal cell death (72, 73). Importantly, we found that ApoE4 and glucose yielded cumulative effects in deteriorating semantic and episodic memory scores and that there was a specific interaction between both factors in predicting episodic memory, indicating that among ApoE4 carriers, glucose appears to worsen episodic memory scores. These cumulative and interacting effects of both glucose and ApoE4 may be explained by the impact of glucose in activating oxidative and inflammatory pathways associated with ApoE4. Moreover, our findings suggest that increased glucose and AGAP could play a role in cognitive disorders, extending the research of Djelti et al., who found that fasting blood glucose was closely linked to spatial memory performance as well hippocampus or septum volumes in grey mouse lemur. (74)

It is interesting to note that we found that ApoE4, increasing age and disorders in anion gap, albumin and glucose have cumulative effects thereby affecting semantic and episodic memory. For example, our neural network model shows that episodic memory is highly predicted

by an interplay among Apo E4, anion gap, glucose and lower albumin. Firstly, this indicates that an age-related mechanism other than that related to glucose and inflammation may be associated with the development of cognitive deficits. One possibility is that age-associated increased levels of eotaxin (CCL-11), which is an endogenous cognition deteriorating chemokine produced by eosinophils, induce neurocognitive deficits in memory through lowered neurogenesis. (75) Secondly, it is probable that in ApoE4 carriers, the central effects of glucose, anion gap and inflammation interact with each other and additionally with the detrimental effects of ApoE4 thereby deteriorating the immune and oxidative stress pathophysiology underpinning cognitive disorders.

In the present study we could not detect significant effects of folic acid on semantic and episodic memory. Neverthless, in some studies, lower levels of folate were associated with cognitive impairments, (76) while intake of folate in MCI and AD patients may improve WLM, MMSE and Constructional Recall test scores, (77) Folate functions as a coenzyme in metabolic pathways in DNA and S-adenosylmethionine (SAM) synthesis (78) and supports neuronal integrity. (79) Chen et al. proposed that supplementing folic acid may improve AD by reducing inflammatory reactions as measured with plasma amyloid beta (Aβ) and mRNA levels of presenilin-1 and tumor necrosis factor α (TNFα). (80) A previous study found a significant association between AIP and 10T-BNT scores in the low frequency words category, (13) while in the current study no significant associations between AIP and episodic or semantic memory scores could be established. Cholesterol and lipids modulate amyloid precursor protein (APP) metabolism, whereby higher cholesterol levels may attenuate APP levels. This reduction in APP metabolism may play a role in nerve cell death and decreased resilience to injuries following attenuated neuroprotective activities (81). It is not clear why lipid metabolism may affect naming but not fluency and recall.

Another major finding of this study is that in aMCI the cumulative effects of AGAP, glucose and ApoE4 were significantly associated with a decline in semantic and episodic memory and that the latter was additionally associated with an interaction between ApoE4 and glucose, indicating that in ApoE4 carriers, glucose further deteriorates episodic memory. Thus, the memory impairments of both AD and aMCI are associated with the same set of biomarkers and similar interaction patterns. Therefore, we may hypothesize that these biomarkers in aMCI may perhaps play a role in the transition from aMCI to AD and/or in shortening the time lag between the diagnosis of aMCI and the onset of dementia. In this respect, Furney et al. reported that the combination of pro-inflammatory cytokines and brain magnetic resonance imaging (MRI) measures is more predictive of the transition from MCI to AD than the ApoE status or clinical data (82). Therefore, future research should examine whether the peripheral biomarkers measured here may predict the conversion from aMCI to AD. Some papers concluded that this conversion occurs independently from the ApoE genotype and amyloid pathology (20) and that injury markers (CSF t-tau and p-tau), but not amyloid biomarkers, are associated with rapid progression from MCI to AD (83).

Limitations

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The results of the current study should be interpreted considering its limitations. First, this study has a cross-sectional design, which does not allow the firm establishment of causal inferences. Second, our statistical prediction of cognitive dysfunction could be incremented by the inclusion of other genes and pathways in the model. Third, confounding variables other than age, sex and education, could have influenced our findings including life-style variables (e.g. physical activity) and nutritional status. Fourth, in our study, biomarkers were assayed only once and one-time increase in those biomarkers would not likely explain the cognitive impairments measured in our study. Nevertheless, given the magnitude of the effects herein described and the knowledge that these biomarkers deteriorate with age (e.g. glucose, inflammation and serum bicarbonate) (84-86) and have known pathophysiological effects (reviewed above), we may argue that our findings could reflect (sub)chronic aberrations in these biomarkers. However, prospective studies are needed with repeated measurements of those biomarkers to further elucidate the relevance of our findings to the progression of neurocognitive impairments and the conversion of aMCI to AD.

Conclusions

The current study found that peripheral blood biomarkers, including anion gap, bicarbonate, albumin and glucose levels, coupled with ApoE4 significantly affected cognitive functions including episodic and semantic memory among elderly with aMCI and AD. Furthermore, these peripheral biomarkers interact with ApoE4 to promote greater memory impairments, suggesting that those biomarkers could interact with the ApoE4-related pathways thereby contributing to the pathophysiology aMCI and AD. Our findings suggest that the transition from aMCI to AD could at least in some cases be associated with significant interactions between ApoE4 and those peripheral blood biomarkers. However, prospective studies are needed to confirm/refute our findings.

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Disclosure Statement

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Author's contributions

All the contributing authors have participated in the manuscript. TS, CT, ST and MM designed the study. TS, CT, ST, DA, IT, SH and PC recruited patients and completed diagnostic interviews and rating scale measurements. MM carried out the statistical analyses. All authors contributed to interpretation of the data and writing of the manuscript. All authors approved the final version of the manuscript.

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