This is an Accepted Manuscript of an article published by Taylor & Francis in Journal of Nutrition in Gerontology and Geriatrics on 29 March 2019, available online: http://www.tandfonline.com/10.1080/21551197.2019.1590287.

The relationship between B-vitamin biomarkers and dietary intake with Apolipoprotein E ϵ4 in Alzheimer's disease.

Journal:	*Journal of Nutrition in Gerontology and Geriatrics*
Manuscript ID	WJNE-2018-0007.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Keywords:	Alzheimer's disease, Dementia, Apolipoprotein E, Homocysteine, Cysteine, folic acid, folate, dietary intake, aging, elderly, nutritional vulnerability

ABSTRACT

The potential for B-vitamins to reduce plasma homocysteine (Hcy) and reduce the risk of Alzheimer's disease (AD) has been described previously. However, the role of Apolipoprotein E ϵ4 (APOE4) in this relationship has not been adequately addressed. This case-control study explored APOE4 genotype in an Australian sample of 63 healthy individuals (female=38; age=76.9±4.7y) and 63 individuals with AD (female=35, age=77.1±5.3y). Findings revealed 55 of 126 participants expressed the APOE4 genotype, with 37 of 126 having both AD and the APOE4 genotype. Analysis revealed an increased likelihood of AD when Hcy levels are >11.0µmol/L (*p*=0.012), Cysteine levels were <255µmol/L (*p*=0.033) and Serum Folate was <22.0nmol/L (*p*=0.003; in males only). In females, dietary intake of total folate <336µg/day (*p*=0.001), natural folate <270µg/day (*p*=0.011) and vitamin B2 <1.12mg/day (*p*=0.028) was associated with an increased AD risk. These results support Hcy, Cys, and SF as useful biomarkers for AD, irrespective of APOE4 genotype and as such should be considered as part of screening and managing risk of AD.

Keywords: Alzheimer disease; Dementia; Aged; Apolipoprotein E; Homocysteine; Cysteine; Folic Acid; Folate; Diet.

INTRODUCTION

Dementia affects an estimated 47.7 million adults worldwide, with the majority of new cases (7.7 million per year) occurring in economically less developed countries (1, 2). Alzheimer's disease (AD) is the most common form of dementia, characterised by a gradually increasing level of cognitive impairment associated with a parallel reduction in quality of life (3). The societal and financial burden of AD are substantial and presents unique challenges for the public health and aged care sectors (4). The aetiology of AD is multifactorial and includes neuronal apoptosis resulting from the aggregation of amyloid-β (Aβ), the formation of intraneuronal neurofibrillary tangles by abnormally hyperphosphorylated tau proteins (5), and a reduction in cerebral glucose metabolism (6). The impact of non-modifiable (including genetics, age, and gender (7)) and modifiable (nutrition, physical activity, and education (8)) risk factors on AD is becoming well recognised. However, the combined effects of modifiable and non-modifiable risk factors for AD pathology is still poorly understood. As quantifiable cognitive decline associated with AD appears approximately 12 years before clinical diagnosis (9), there is an urgent public health need to identify those at high risk and intervene to slow progression and prevent the onset of AD.

Nutrition can both positively and negatively influence cognition in the elderly, as evidenced by the association of B-vitamin deficiency with AD and other dementias (8, 10, 11). The B-vitamins include folic acid (both synthetic and natural forms), vitamin B2 (riboflavin), vitamin B6 and vitamin B12 (including its synthetic form, cyanocobalamin) as essential precursors for coenzymes involved in the one-carbon metabolism pathway of homocysteine (Hcy), and thiol biosynthesis (12). Thiols are plasma sulfhydryl-containing amino acids (Hcy, cysteine [Cys], cysteinyl-glycine [CysGly] and glutathione [GSH]) that play a vital role in cardiovascular health and cognition (13, 14). Elevated Hcy levels were identified as a strong predictor of incident AD

(8), while adequate dietary intake of folate and vitamin B12 (B12) plays a major role in the methylation and transsulfuration pathways and contribute to the maintenance of reduced Hcy levels (15). Hence, Hcy, folate, and B12 have been identified as important blood-based biomarkers of nutritional status and AD risk (8, 10).

Elevated levels of plasma Hcy along with low levels of folic acid and B12 are prevalent in individuals with AD (16). Elevated plasma Hcy is involved in AD through the promotion of oxidative stress, leading to neuronal damage and impairment of Blood-Brain Barrier (BBB) permeability (17). While folic acid and B12 possess antioxidant properties with the capacity to counteract such damage (16), the oxidative stress associated with AD pathology may also be due to Aβ-induced oxidative stress which increases plasma Hcy levels by depleting 5-methyltetrahydrofolate (5-MTHF) (16). SF is also proposed as a useful biomarker of Aβ accumulation (18). Nevertheless, Hcy is a known independent risk factor for the development of AD, as is low serum folate (SF) (19). The Framingham Study (20) identified plasma Hcy levels greater than 14 µmol/L to be associated with doubled risk of developing AD. However, despite consistent reductions in Hcy from various formulations of B-vitamin supplementation, controversy remains surrounding their ability to prevent or reduce symptoms of cognitive decline or incidence of dementia due to heterogeneity of study design and the likelihood that B-vitamins may benefit those with low blood levels prior to the intervention (21, 22). Noticeable cognitive decline associated with AD presents several years after the onset of the related pathologies, and it is plausible that B-vitamin interventions in older adults are implemented too late to offer protection against further decline or even symptomatic relief due to the damaging effects of decades of elevated plasma Hcy and nutritional deficiency (21, 23).

Apolipoprotein E (APOE) plays an integral role in the brain through its support of synaptic plasticity, cholesterol metabolism, and the management of neuroinflammation (24). The

Apolipoprotein E ϵ4 (APOE4) allele is the most common known genetic risk factor for AD, providing 3-fold increased odds in individuals with one copy, and approximately 15-fold increase in those with two copies (25). The allele has been reported to lower the age of onset of AD (24), is an established risk factor for coronary heart disease (24), and is a genetic indicator of reduced life expectancy (26). A lower concentration of APOE is associated with impaired clearance of Aβ from the brain, with even more pronounced effects in APOE4 carriers with AD (27). Lower plasma APOE concentration is also associated with smaller hippocampal size in individuals with AD, particularly in APOE4 carriers (28). The effect of diet and nutrient intake on APOE levels is not currently well defined, with dietary interventions such as the Mediterranean diet (MD) reporting mixed results in APOE4 carriers (29, 30). In APOE4 carriers, adherence to the MD (traditionally rich in B-vitamins) is associated with better cognitive performance compared to a contemporary Western Diet (30). However, in a study of executive function, findings suggested that an MD based intervention may not be as successful in APOE4 carriers compared with non-carriers (29). Higher overall dietary energy intake increases the risk of AD in APOE4 carriers (31), while cognitive performance and hippocampal APOE can be moderated by dietary fat intake (32). As APOE4 carriers may have a genetic disposition to increased fatty acid mobilisation and utilisation, significant questions remain surrounding the optimal dietary recommendations for APOE4 carriers (33).

The relationship between one-carbon metabolism, B-vitamin status, and APOE4 genotype in AD has only recently started to receive significant attention (34). As APOE4 forms a weak complex with Aβ that may result in Aβ accumulation (35), investigation of a potential increased AD risk associated with APOE4 and poor B-vitamin status is warranted. To date, several studies have failed to find an increased risk of cognitive dysfunction in APOE4 carriers relative to plasma thiol status (36-38). However, the association between low serum B12 status and impaired cognition in APOE4 carriers has been established (38-40). The present study aimed to investigate the role of biomarkers of B-vitamin status including Hcy, and dietary B-vitamin intake in sporadic AD relative to APOE4 genotype focused in a case-control study of healthy elderly Australians and individuals clinically diagnosed with AD. The suitability of biomarkers of B-vitamin status and dietary B-vitamin intake as diagnostic covariates was also assessed.

METHODS

Study design and subject details

The study was designed as a pair-matching case-control study with the cases and controls recruited from two distinct cohort studies. Between 2007 and 2008, 126 elderly adults (73 females and 53 males) aged between 65 and 83 years who resided in the New South Wales (NSW) Central Coast region of Australia were recruited as part of a comparison pilot study for retirement living. The AD cohort consisted of 63 individuals (35 females and 28 males; mean age 77.1±5.3 years), recruited over a similar time period for a folate-related study and clinically diagnosed with AD using the criteria set by the National Institute of Neurological and Communicative Disorders and Stroke and by the Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA). The healthy control cohort consisted of 63 community-dwelling individuals (38 females and 25 males; mean age 76.9±4.7 years) from a high socio-economic area retirement village. Each control subject was matched with a dementia (case) based on age (between 65-83 years) (±1 year). To determine that the two groups were matched by age, and different based on MMSE score, the student’s *t*-test was conducted. The samples were selected randomly from each of the cohorts and individuals were excluded if they fell outside the age range or if a suitable matching control subject was not identified. Matching by gender was attempted, but not possible because of the differences in sex distribution between the two cohorts and the size of the cohorts. The AD group, described above, received a diagnosis from practising neurologists associated with the study during visits to NSW Central Coast clinical practices. All participants had completed at least seven years of formal education.

Ethics approval: Northern Sydney Central Coast Health Committee (approval numbers 04/19 & 06/224 for the control and AD groups respectively) and the University of Newcastle Human Research Ethics Committee (approval numbers H-782-0304 & H-2008-0418 for the control and AD groups respectively) apply. The University of Newcastle and the University of Canberra, Human Research Ethics Committees had formal reciprocal arrangements for approval to use study data and samples.

Cognitive testing

During the clinical assessment, all participants completed a face-to-face MMSE (41), self-completed a Hospital Anxiety and Depression Scale score (HADS) (42), and the neurologist obtained a brief medical history. Written informed consent for participation in this study, from participants that scored less than 24 on MMSE, was obtained by a registered proxy who was assessed as having the necessary cognitive capacity (41).

Dietary intake of B-vitamins

All participants completed estimation of daily intake of nutrients during an interviewer-administered validated food frequency questionnaire (FFQ), (23, 42, 43) adapted from the

Commonwealth Scientific and Industrial Research Organization version (CSIRO) (44). During the interview, participants disclosed current supplements use, if any. If a participant was unable to provide adequate responses, the participant's carer was asked to provide the food intake information. The FFQ data were analysed using Foodworks^{TM} v3.02 Professional software (Xyris Software, QLD, Australia). This software incorporates most food items consumed by Australians at the time of data collection. It is important to note that all data and samples in this study were collected before mandatory folic acid fortification was introduced in Australia in late 2009.

Blood collection and processing

Blood was collected (approximately 20mL) from each participant by phlebotomists following an overnight fast of at least 10 hours duration. Plasma samples were collected in Lithium Heparin tubes, and serum samples were collected in tubes containing a clot activator. Samples were processed and stored at -80°C until analysis (43).

Biochemical assays

Total plasma thiol levels (Hcy, Cys, Cys-Gly, and GSH) were determined by High-Performance Liquid Chromatography (HPLC) with a fluorescence detector after 4-Fluoro-7-sulfobenzofurazan (SBD-F) ammonium salt derivatization at the Molecular Nutrition Laboratories at the University of Newcastle (Ourimbah, NSW, Australia) (42, 45). The red blood cell folate (RBCF), SF and serum B12 were measured using a standardized automated Access Immunoassay System as part of routine analysis at either the Institute of Clinical Pathology and Medical Research (ICPMR) at Westmead Hospital (Sydney, NSW, Australia) or the Gosford Hospital Pathology Laboratory

(Gosford, NSW, Australia). APOE4 analysis was performed following the manufacturer's instructions at the University of Canberra (Canberra, ACT, Australia) using commercially available Enzyme-linked Immunosorbent Assay (ELISA) kits to determine plasma APOE (SKU: ab20874. Abcam, Cambridge, United Kingdom) and serum APOE4 (SKU: K4699-100. Biovision, Milpitas, CA, United States) concentrations. Serum samples that fell within the detection range were determined to be positive for APOE4 genotype. Each kit was verified using the sample of a known APOE genotype collected before analysis by a qualified nurse. The fresh sample was immediately processed and analysed using standardised procedures and stored appropriately at -80°C for inter- and intra-assay variabilities. Inter- and intra- assay coefficients of variation for both kits were less than 10%.

Statistical analysis

Power calculations were based on a-priori statistical power analysis. A sample size of 59 AD patients and 59 age- and sex-matched healthy subjects (+10% for missing values) was established as adequate in order to evaluate two-sided odds ratios equal to 1.20 and differences at least 20% in primary (MMSE) and secondary (Hcy and SF levels) outcomes, achieving statistical power greater than 0.80 at 0.05 probability level (*p*-value). All variables were examined prior to analysis to determine suitability for parametric or non-parametric methods using histograms, and both Kolmogorov-Smirnov and Shapiro-Wilk tests of normality. Descriptive statistics for normally distributed continuous variables are reported as a mean ± standard deviation, while non-normally distributed variables are reported as median values (1st, 3rd quartiles). Student's *t*-test for independent samples was used to evaluate differences between groups for normally distributed variables, and the Mann-Whitney test was used for non-parametric variables. Chi-square test of independence was performed to examine the association between AD status and MMSE categories. A receiver operator characteristic (ROC) curve was used to test the discriminatory power of variables and area under the curve (AUC) of biomarkers and dietary intake relative to AD and APOE4 genotype in the study sample. Predictor values based on Swets (46), distinguish predictive accuracy as: '*low*' ($0.500 < AUC \leq 0.700$); '*moderate to high*' ($0.700 < AUC \leq 0.900$); and '*very high to perfect*' ($0.900 < AUC \leq 1.00$). Youden index was calculated to determine the optimal cut-off points. Due to the variability of the number of decimal points reported in the biochemical assay results, we presented numerical data as suggested by Cole (47) throughout this manuscript. The level of significance was defined as *alpha*< 0.05 and no adjustments were made for multiple comparisons. All statistical analysis was performed using IBM SPSS version 23.0 207 (Armonk, NY: IBM Corp).

RESULTS

The total sample included 126 individuals, and consisted of 38 females in the healthy control group and 35 in the AD group. A flowchart of the two groups is presented in Figure 1. The median age in the control group was 78.0 years (74.0, 81.0) and the median age was 79.0 years (73.0, 82.0) in the AD group. Analysis of group differences between (*n*=63) individuals clinically diagnosed with AD and (*n*=63) community-dwelling controls free of cognitive impairment indicated that the clinical status of AD was significantly associated with HADS depression score (Control: 3.00 [2.00, 5.00]; AD: 8.00 [3.00, 11.00]; $p < 0.001$) and HADS total score (Control: 8.00 [5.00, 13.0]; AD: 12.0 [5.00, 18.0]; $p = 0.026$). A chi-square test of independence was performed to examine the relation between the MMSE score of both groups by MMSE category (48). The relation between variables indicated a lower MMSE score in the AD group (X^2 [3, $n = 126$] = 113.2, $p < 0.001$). Baseline characteristics of the control and AD groups are indicated in Table 1.

Dietary intake of B-vitamins

Overall, there was no significant difference in dietary B-vitamin intake between the control and AD groups ($p > 0.05$) except for dietary folate intake in females. Females diagnosed with AD had significantly lower ($p < 0.001$) intake of total folate in the control group (439 ± 172 µg) compared with the AD group (321 ± 87.6 µg). Natural folate intake was also higher ($p = 0.007$) in the control group (308 ± 103 µg) compared with the AD group (245 ± 78.1 µg). Also, levels of estimated dietary intake of B-vitamins (Table 2) did not vary between groups or sub-groups for vitamins B1 (thiamine), B2, B3 and synthetic folate (all, $p > 0.05$).

Plasma thiols and other blood biomarkers

The plasma thiol and blood biomarker analysis revealed differences between groups in SF, Hcy and CysGly (Table 3). SF concentration was significantly lower ($p = 0.008$) in all AD participants (18.0 nmol/L [13.0, 29.0]) compared with controls (24.0 nmol/L [19.0, 36.0]). In males with AD (14.5 nmol/L [12.0, 21.8]) , SF was much lower ($p = 0.003$) than the male controls (23.0 nmol/L [18.0, 31.0]). The Hcy level was also significantly higher in all individuals with AD (Control: 9.39 ± 2.60 µmol/L; AD: 11.61 ± 4.98 µmol/L; $p = 0.002$), females (Control: 9.18 ± 2.70 µmol/L; AD: 11.6 ± 5.87 µmol/L; $p = 0.028$) and males (Control: 9.70 ± 2.46 µmol/L; AD: 11.6 ± 3.69 µmol/L; $p = 0.033$). The plasma CysGly level was lower ($p = 0.046$) only in females diagnosed with AD (21.8 µmol/L [18.9, 25.0]) compared with Control (23.6 µmol/L [21.4, 26.3]), while no other differences were observed between groups for CysGly (all, $p > 0.05$). The biomarkers did not vary between groups or gender for serum B12, RBCF, Cys, GSH and plasma APOE expression (all, $p > 0.05$).

Plasma thiols and blood biomarkers by APOE genotype

In total, 55 participants (43.7%) expressed the APOE4 genotype, and 37 participants (29.4%) were clinically diagnosed with AD and possessed the APOE4 genotype. After examining differences between groups for APOE genotype and gender, there were no differences for serum B12, RBCF, Cys, CysGly, and GSH (all, $p > 0.05$). The SF was significantly lower in males possessing APOE4 when compared to males without (APOE4-: 21.5 nmol/L [16.3, 28.8]; APOE4+: 15.0 nmol/L [11.0, 23.0]; $p = 0.036$) and lower in APOE4+ individuals with AD (Control/APOE4-: 23.0 nmol/L [11.0, 23.0]; AD/APOE+: 18.0 nmol/L [12.0, 27.5]; $p = 0.035$). The plasma Hcy level was higher ($p = 0.012$) in the APOE4+ group with AD (11.2 ± 3.98 µmol/L) compared with APOE4- controls (9.33 ± 2.19 µmol/L), but not in the other groups (all, $p > 0.05$). Additionally, plasma APOE concentration was significantly lower ($p = 0.042$) in participants with the APOE4 genotype compared to those without, although this relationship was mainly due to the female group (APOE4-: 57.4 µg/mL [46.8, 69.9]; APOE4+: 46.7 µg/mL [38.2, 64.7]; $p = 0.041$).

Predictor value of plasma thiols, blood biomarkers and B-vitamin status by receiver operating characteristic curve

To evaluate the predictor value of the biomarkers using our case-control data set, we utilised ROC curves to determine the diagnostic potential of the covariates relative to AD status. Using these models, we estimated the threshold for diagnostic ability and predictor value and presented the statistically significant values ($p < 0.05$) in Table 5. The cut-off predictor values of significant covariates are presented in Table 5.

The analysis revealed that plasma Hcy levels greater than 11.0 µmol/L were associated with an AD diagnosis (AUC [95% CI]: 0.629, $p = 0.012$). This association was not significant in the APOE4+ with AD subgroup ($p = 0.058$). Plasma Cys levels of under 255 µmol/L and 253 µmol/L represented an increased chance of an AD diagnosis (AUC [95% CI]: 0.610, $p = 0.033$) and similarly in the APOE4+ with AD subgroup (AUC [95% CI]: 0.629, $p = 0.045$), respectively. SF under 18.0 nmol/L was also associated with increased chance of AD diagnosis in all with AD (AUC [95% CI]: 0.637, $p = 0.008$) and APOE4+ with AD (AUC [95% CI]: 0.636, $p = 0.035$). In males, SF levels under 22.0 nmol/L and 22.5 nmol/L were associated with increased likelihood of AD diagnosis in the AD group and APOE4+ with AD sub-group, respectively. This level of SF in males was associated with a moderate to high ability to predict an AD diagnosis (AUC [95% CI]: 0.735, $p = 0.003$) (Figure 2A). Similar ability of SF was found in the APOE4+ with AD sub-group (AUC [95% CI]: 0.819, $p = 0.002$) (Figure 2B).

Different effects of dietary intake and risks were seen between genders, wherein females, average daily dietary intake of total folate, natural folate, and vitamin B2 was associated with an increase in the likelihood of an AD diagnosis. Total folate intake of under 336 µg/day increased the chance of an AD diagnosis (AUC [95% CI]: 0.736, $p = 0.001$) (Figure 2C). Natural folate intake of less than 270 µg/day also increased the likelihood of an AD diagnosis (AUC [95% CI]: 0.687, $p = 0.011$). In addition, vitamin B2 intake of under 1.12 mg/day was associated with increased likelihood of AD diagnosis (AUC [95% CI]: 0.661, $p = 0.028$). These covariates were not significantly associated with an AD diagnosis in the APOE4+ with AD subgroup (all, $p > 0.05$).

DISCUSSION

This study represents an exploration of the potential associations between biomarkers of B-vitamin status, dietary intake of B-vitamins and APOE4 genotype in an Australian AD cohort. The data suggested that biomarkers of B-vitamin status, particularly Hcy, Cys, and SF, are significantly associated with AD as potential diagnostic tools in an elderly cohort. Furthermore, both increased Hcy levels and APOE4 expression were strongly associated with AD. This is consistent with the findings of Miwa et al. (49) that show both Hcy and APOE4 contribute to the development of AD. Novel findings surrounding sex differences, folate levels, and APOE4 were also identified. Reduced dietary folate intake in elderly females with AD may function as a possible predictor for AD, regardless of APOE genotype. In males with AD, the SF was lower compared with healthy males and could be used as a predictor variable for AD.

The link between increased plasma Hcy levels and AD is well established, however, to our knowledge, only a few studies (39, 50, 51) have investigated the relationship of Hcy with APOE4 in humans. Our study identified that 43.7% of the total participants possessed the APOE4 genotype and 29.4% of all participants had both the APOE4 genotype and AD. While these figures may be considered high relative to worldwide prevalence (25), an Australian study (52) previously reported a frequency of 53% in individuals (*n* = 80) with both the APOE genotype and AD in a clinic-based sample. Increased plasma Hcy levels were found across groups, which was associated with AD status but not the APOE4 genotype. Specifically, plasma Hcy levels over 11.0 µmol/L were identified to be a significant predictor variable for AD, but not in individuals with both the AD and the APOE4 genotype. This Hcy threshold aligns well with previous findings that low dietary B-vitamin intake and high Hcy levels may be used as predictors of cognitive decline (11, 15). APOE4 is the most widely accepted, and potentially most potent genetic risk factor for AD, while hyperhomocysteinemia may be a significant biomarker for those without the APOE4 genotype (17). Minagawa et al. (17) proposed that the thiol component present in plasma Hcy interacts with Cys residues of the more abundant Apolipoprotein E ϵ3 (APOE3), yet it does not appear to interact with APOE4. The interaction between Hcy and APOE3 interferes with dimerisation and impairs high-density lipoprotein (HDL) production. As HDL function is typically enhanced in carriers of APOE3 compared to those with APOE4 (53), this mechanism may explain an increased risk of elevated Hcy in APOE3 carriers. In addition, plasma Hcy may decrease APOE expression (54), potentially contributing to the reduced clearance of Aβ independent of APOE genotype, which could add to oxidative stress and increase the risk of AD. These potential mechanisms provide plausible explanations for the finding in this analysis, proposing that Hcy represent an important tool for measuring AD regardless of APOE genotype.

These data support an independent association between plasma Hcy levels and APOE4 in AD, alongside sex differences explored as predictors of an AD diagnosis. The association between inadequate total dietary folate intake and AD risk is well established (11, 19, 55, 56). In this current study, the predictor abilities of total folate (over 336 μg/day), natural folate (over 270 μg/day), and vitamin B2 (over 1.12 mg/day) were associated with a reduced chance of an AD diagnosis in females. However, these associations were not significant in individuals with both AD and the APOE4 genotype. Further findings emphasise the importance of the inclusion of dietary natural folate and vitamin B2 as a protective strategy for AD in females as it may offer protection in the prodromal phase of the disease development.

The only biomarker in our analysis to reveal moderate to high predictor value was SF in males, however, our findings support the importance of the transsulfuration pathway in AD aetiology. Our sample confirms elevated Hcy as an important risk factor for AD, and also found lower Cys to be a potential predictor variable. Cys is a component of glutathione, an important endogenous antioxidant in the brain, and while no differences were observed with GSH in our sample, low Cys has been linked to mortality and frailty in older adults (57). The reaction of elevated Hcy to Cys is dependent on dietary intake of and hepatic conversion of vitamin B6 into pyridoxal-5-pyrophospate, and deficiencies in B6 can lead to increased Hcy. However, we do not have vitamin B6 data to further assess this relationship.

In this study, male participants diagnosed with AD were estimated to be consuming 468 µg/day of total folate compared with 435 µg/day in HC, yet SF was lower in males with AD. In men, SF greater than 22.0 nmol/L was a diagnostic predictor of the reduced likelihood of AD in the AD group. No differences in RBCF were observed ($p > 0.05$) for either gender. The difference in folate consumption between males and females with AD may at least be partially explained by higher energy intake in the males. Although reduced SF in males is not a unique finding (11), more research is required to explore the potential sex differences between total folate intake and SF levels, as well as the risk of lower Cys.

Mandatory fortification of key foods with synthetic folate, together with other factors such as education, may be contributing to the decreasing AD incidences in more developed, but not developing, countries (58). However, countries such as the United Kingdom and New Zealand, and many developing countries are still considering the merits of mandatory folic acid fortification. In Australia, folic acid fortification commenced in 2009 and was shown to reduce plasma Hcy levels and incidence of hyperhomocysteinemia while increasing SF and RBCF (23). Beckett et al. (23) also found an increased SF and RBCF levels; however, the authors attributed this to the effect of synthetic folate fortification, and not natural folate consumption. Long-term supplementation of older adults with B-vitamins has been shown to reduce Hcy levels, albeit there is mixed evidence surrounding the delay of cognitive decline (21). However, one RCT has shown benefit to B-vitamin supplementation in individuals with mild cognitive impairment with baseline Hcy of above 11.3 µmol/L (59), in accordance with our threshold value of the likelihood of AD diagnoses with Hcy above 11.0 µmol/L. Benefits of folic acid fortification may be due to increased consumption during the prodromal phase of AD development as both short and long-term trials of folic acid supplementation in younger individuals have reported improved cognitive outcomes (60, 61). Therefore, it is plausible that there could be a precise therapeutic dose of folic acid necessary to prevent cognitive decline in older adults. However, larger scale RCTs in at-risk individuals are required before general supplementation recommendations are considered, as too much folate may be detrimental (56). Combined data from three cohorts have reported poorer cognitive function in elderly with low B12 status and high RBCF, further complicating the potential of a recommended intake of B-vitamins in individuals at-risk (62).

The current study included a case-control design and ROC curve analysis of relevant biomarkers of B-vitamin status and dietary intake of B-vitamins. Implementation of ROC curve analysis is considered ideal for case-control studies, particularly when comparing disease susceptibility genes in AD, such as APOE (63). This study revealed a relatively high number of individuals expressing the APOE4 genotype, making it suitable for a robust comparative analysis. Biomarkers in this study were blood-based, and the majority of them can be tested at pathology labs in Australia. The use of CSF-based biomarkers can be relatively reliable in predicting AD diagnosis but necessitates an intrusive lumbar puncture that is considered too invasive for routine disease screening. However, blood work is routinely practised, cost-effective, and is well tolerated in the community for the basis of health management and screening for disease. Hence the ability to establish AD risk using blood biomarkers is a promising approach for early detection and screening for AD. The APOE4 is a strong genetic risk factor, yet not all individuals who develop AD carry the APOE4 allele, particularly those with only one copy. Therefore, studies that investigate multiple biomarkers may allow increased precision in the prediction of AD risk and could benefit individuals and communities alike. Interestingly, a recent small study (*n* = 17) using a ROC curve analysis found SF and red blood cell haemoglobin to be useful biomarkers of Aβ accumulation in the brain with more sensitivity and specificity than APOE genotype or folate alone (18). Future research targeting a combination of biomarkers namely APOE, Hcy, and SF in larger samples is required to support a clinical diagnosis of AD using a simple blood test. Future studies should also include an analysis of omega-3 fatty acid status due to a possible beneficial synergy with B-vitamins in mild cognitive impairment (64).

One of the limitations of the study is the use of estimated dietary data by using FFQ, as this method is prone to the underestimation of energy intakes and omittance of unhealthy eating habits (65). The FoodWorks^{TM} Professional v3.02 software contains historical databases, before the mandatory folate fortification of wheat flour in Australia and provides incomplete information for the B6 and B12 content of many foods. For this reason, these values were not analysed as dietary intake. However, the values of serum B12 in our sample did not differ between groups and were above that of the recommended clinical thresholds set in Australia. In females, Folate intake below the recommended dietary intake is associated with an increased risk of mild cognitive impairment and probable dementia, but no such association was identified with B12 intake in a large prospective longitudinal cohort (55). However, the analysis of our sample allowed for the estimation of dietary folate intake before mandatory fortification of folate was introduced in Australia. Our study provides valuable insight into folate status for countries considering mandatory folic acid fortification. Our study was also unable to determine how many copies of the APOE4 allele each participant possessed. Therefore, valuable analysis discriminating between individuals possessing one or two copies of the APOE4 allele was not possible. We were also unable to match cases and control by sex; however, the distribution of sexes is similar. Furthermore, only limited socio-demographic data was available for this retrospective analysis. Finally, the retrospective nature of this study and its cross-sectional nature can only identify associations, and as previously stated would require a larger prospective study to confirm its findings.

In conclusion, our findings do not support an association between biomarkers of B-vitamin status and dietary intake of B-vitamins, relative to APOE4 genotype in a case-control study of healthy and clinically diagnosed individuals with AD. We have uncovered associations that may aid in estimation of an AD diagnosis through the use of plasma Hcy levels, SF intake in males, and dietary intake of folate and vitamin B2 in females. Future studies using larger sample sizes may aid in further defining these relationships and their potential role in the screening of AD.

TAKE AWAY POINTS

- In this case-control study of elderly Australians, the presence of the Apolipoprotein E ϵ4 genotype was not associated with B-vitamin biomarkers of Alzheimer’s disease.
- Elevated blood homocysteine, low cysteine, and low serum folate was associated with the likelihood of an Alzheimer’s diagnosis.
- Lower dietary folate intake in females was associated with the likelihood of an Alzheimer’s diagnosis.
- The effect of fortification of the Australian diet with folate (which began after this data was collected) on dietary folate intake and serum folate leaves and subsequent Alzheimer’s risk warrants further study.

Funding details: This work was supported by the funding from the University of Canberra Health Research Institute (UCHRI) – Research and support development program. Nathan M D'Cunha is supported by the Dementia Australia Research Foundation PhD Scholarship.

Disclosure statement: All authors declare no conflict of interest.

Acknowledgments: The authors would like to thank Dr Jackson Thomas for his comments and proof-reading of the manuscript.

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FIGURES

Figure 1.

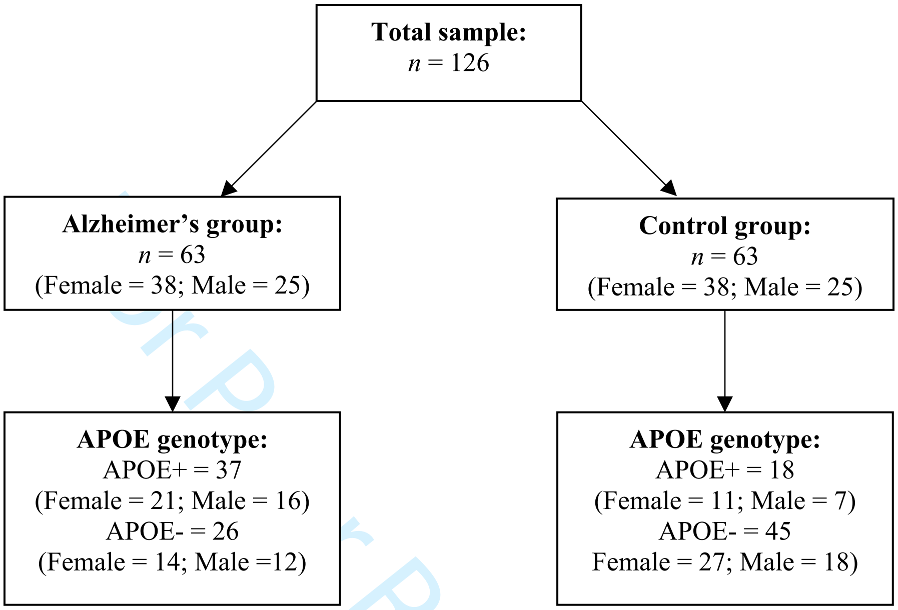
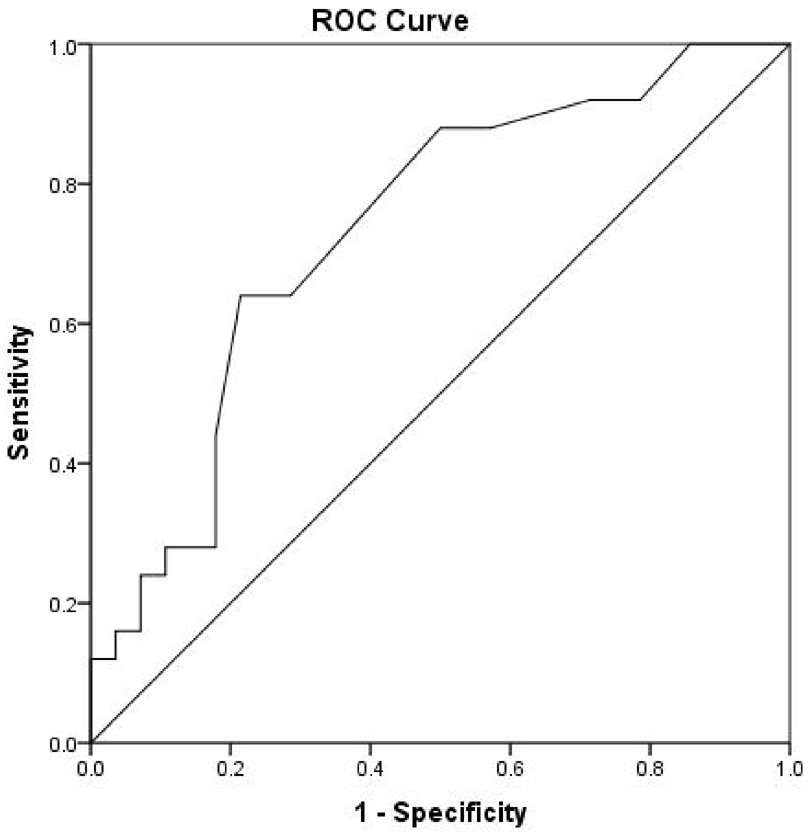


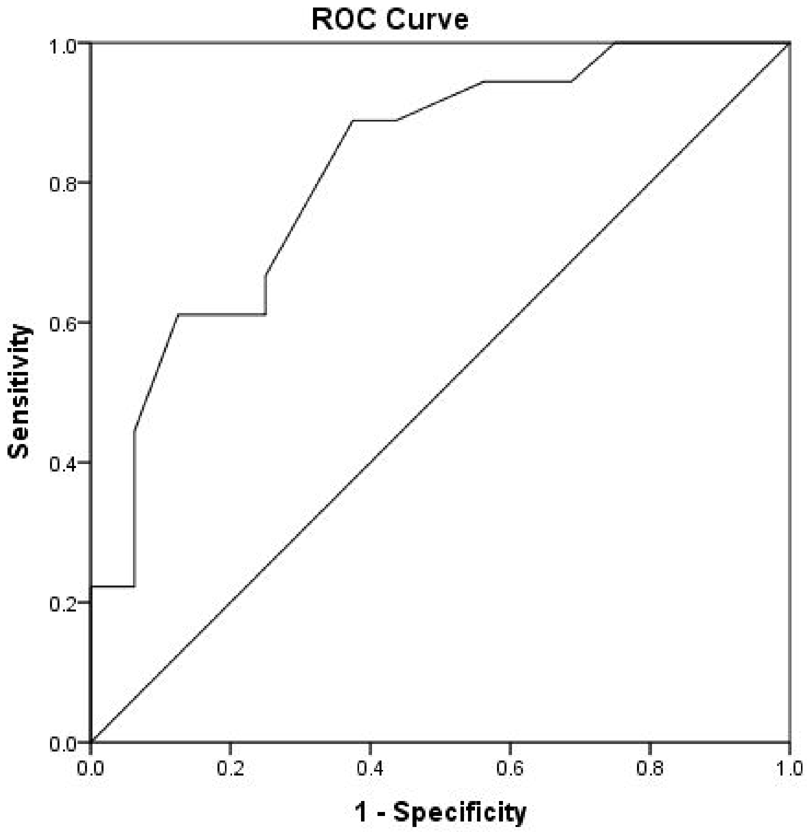
Figure 2.

A.



Diagonal segments are produced by ties.

B.



Diagonal segments are produced by ties.

C.

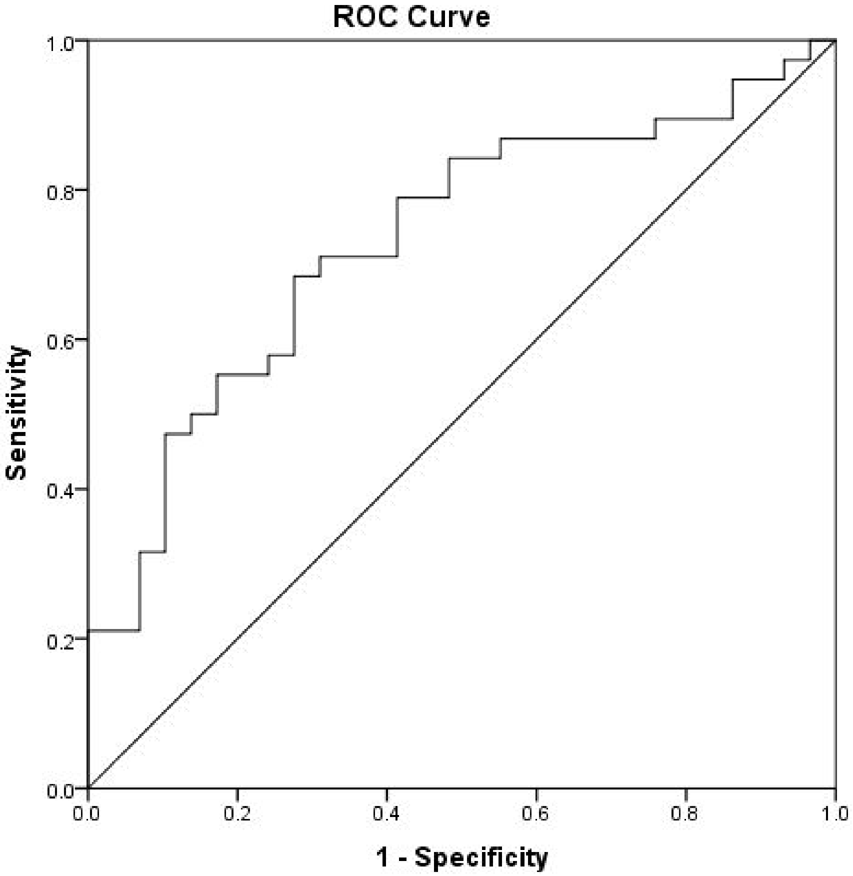

ROC Curve
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0
0.0
0.2
0.4
0.6
0.8
1.0

FIGURE LEGEND

Figure 1.

Flow diagram showing the number of participants in each of the groups, gender, and Apolipoprotein E genotype.

Figure 2.

Receiver-operating characteristic (ROC) curves representing models with the highest predictive ability of all biomarkers and dietary intake. The models differentiate between: A: Serum folate in males with Alzheimer's disease (AD) and male healthy controls (Area under the curve [AUC]: 95% Confidence interval [CI]= 0.735, *p*=0.003); B: Serum folate in males with AD & apolipoprotein E ϵ4 genotype and male controls (AUC: 95% CI= 0.819, *p*=0.002); C: Dietary total folate intake in females with AD and female controls (AUC: 95% CI= 0.736, *p*=0.001). Significance was measured as *p*<0.05.

Table 1. Socio-demographic and neuropsychological characteristics per Alzheimer's disease status *(n=126)*

	Control	**AD**	***p***
n	63	63	
Gender (female)	38 (60.3%)	35 (55.6%)	0.588
Age (years)	78.0 (74.0, 81.0)	79.0 (73.0, 82.0)	0.806
HADS Anxiety	5.00 (2.00, 7.00)	4.00 (1.00, 7.00)	0.098
HADS Depression	3..00 (2.00, 5.00)	8.00 (3.00, 11.0)	<0.001
HADS Total	8.00 (5.00, 13.0)	12.0 (5.00, 18.0)	0.026
MMSE	29.0 (28.0, 30.0)	22.0 (18.0, 24.0)	<0.001
MMSE (*n*)			<0.001[a]
- >27	59 (93.7%)	0 (0%)	
- 24-26	4 (6.3%)	16 (25.4%)	
- 18-23	0 (0%)	33 (52.4%)	
- <17	0 (0%)	14 (22.2%)	

Notes: Non-normally distributed variables are presented as median (interquartile range). Categorical variables are presented as frequencies (relative frequencies). [a] Relationship between AD status and lower MMSE was significant according to Chi-square test of Independence (X^2 [3, N=126] = 113.2, p<0.001). Outcomes were considered to be statistically significant at p<0.05. AD: Alzheimer's disease; HADS: Hospital Anxiety and Depression Scale; MMSE: Mini-mental state examination.

Table 2. Estimated B-vitamin consumption from food frequency questionnaire per Alzheimer's disease status and per gender (*n=126*)

	All		**Female**		**Male**		**Control APOE-ε4-**	**AD APOE-ε4+**	**Control APOE-ε4+**	**AD APOE-ε4-**
	Control	**AD**	**Control**	**AD**	**Control**	**AD**				
n	63	63	38	35	25	28	45	37	18	26
Thiamin (mg)	1.63 ± 0.455	1.63 ± 0.532	1.59 ± 0.477	1.51 ±0.553	1.68 ± 0.426	1.76 ± 0.489	1.65 ± 0.464	1.63 ± 0.412	1.60 ± 0.444	1.63 ± 0.686
Riboflavin (mg)	2.18 ± 0.640	2.09 ± 0.903	2.23 ± 0.676	1.86 ± 0.857	2.11 ± 0.591	2.36 ± 0.893	2.23 ± 0.638	1.63 ± 0.415	2.05 ± 0.644	2.09 ± 1.13
Niacin (mg)	21.6 ± 5.70	21.2 ± 7.22	20.8 ± 6.59	19.08 ± 6.67	22.9 ± 3.97	23.5 ± 7.22	21.4 ± 22.7	22.7 ± 7.71	22.2 ± 5.66	18.9 ± 5.85
Niacin eq. (mg)	38.5 ± 9.50	37.3 ± 11.5	37.8 ± 11.0	33.8 ± 10.2	39.43 ± 7.09	41.1 ± 11.9	38.2 ± 9.61	39.5 ± 12.1	39.1 ± 9.48	33.9 ± 10.0
Total Folate (μg)	438 ± 175	393 ± 160	439 ± 172**	321 ± 87.6**	435 ± 182	468 ± 186	423 ± 172	376 ± 102	474 ± 182	417 ±223
Natural Folate (μg)	307 ± 94.7	278 ± 91.2	308 ± 103**	245 ± 78.1**	305 ± 83.0	313 ± 92.1	306 ± 99.9	285 ± 94.5	308 ± 82.6	265 ± 86.6
Synthetic Folate (μg)	87.6 (28.5, 170)	99.9 (59.9, 107)	70.9 (17.4, 185)	89.9 (15.1, 99.9)	92.7 (33.2, 144)	99.9 (69.0, 220)	60.0 (16.8, 152)	99.9 (70.8, 99.9)	104 (50.0, 300)	99.0 (15.6, 296)

Notes: Estimations are per day. Continuous normally distributed variables (Thiamin, riboflavin, niacin, niacin eq, total folate, and natural folate) are expressed as mean ± standard deviation. Not normally distributed variable (Synthetic folate) is displayed as median (1st, 3rd quartile). Outcomes were considered to be statistically significant at $p < 0.05$; * represents $p < 0.05$; ** represents $p < 0.01$. AD: Alzheimer's disease; APOE: Apolipoprotein E; eq=equivalents.

Table 3. Plasma Thiols and blood biomarkers per gender and Alzheimer's disease status *(n=126)*

	All			**Female**			**Male**		
	Control	**AD**	***p***	**Control**	**AD**	***p***	**Control**	**AD**	***p***
n	63	63		38	35		25	28	
Serum B12 (nmol/L)	246 (200, 298)	217 (171, 290)	0.98	250 (201, 325)	221 (179, 291)	0.148	243 (193, 291)	212 (167, 289)	0.383
Serum folate (nmol/L)	24.0 (19.0, 36.0)	18.0 (13.0, 29.0)	0.008	25.5 (18.8, 39.3)	20.0 (14.0, 44.0)	0.352	23 (18.0, 31.0)	14.5 (12.0, 21.8)	0.003
RBC folate (nmol/L)	898 ± 324	887 ± 493	0.878	896 ± 348	887 ± 519	0.925	902 ± 291	887 ± 468	0.896
Homocysteine (µmol/L)	9.39 ± 2.60	11.6 ± 4.98	0.002	9.18 ±2.70	11.6 ± 5.87	0.028	9.70 ± 2.46	11.6 ± 3.69	0.033
Cysteine (µmol/L)	275 ± 25.4	266 ± 44.2	0.175	279 ± 25.4	269 ± 50.3	0.271	269 ± 35.8	263.4 ±35.8	0.514
Cysteinyl glycine (µmol/L)	24.0 (21.5, 26.9)	23.0 (20,1, 25.4)	0.193	23.6 (21.4, 26.3)	21.8 (18.9, 25.0)	0.046	24.6 (21.3, 27.6)	24.2 (20.8, 28.0)	0.986
Glutathione (µmol/L)	10.5 (8.44, 12.3)	10.9 (8.35, 13.9)	0.251	9.55 (8.39, 12.0)	10.9 (9.07, 13.4)	0.178	10.9 (8.42, 12.6)	11.0 (8.27, 14.4)	0.748
APOE (µg/mL)	51.5 (41.1, 64.2)	50.9 (38.6, 67.1)	0.466	56.8 (46.7, 68.5)	50.9 (35.6, 67.1)	0.107	46.9 (40.2, 61.9)	50.1 (40.3, 67.3)	0.354

Notes: Continuous normally distributed variables (RBC folate, homocysteine, and cysteine) are expressed as mean ± standard deviation. Non-normally distributed variables (serum B12, serum folate, cysteinyl-glycine, glutathione, APOE) are displayed as median (interquartile range). Outcomes were considered to be statistically significant at $p<0.05$. AD: Alzheimer's disease, APOE: Apolipoprotein E; RBC: Red blood cell.

Table 4. Plasma Thiols and Biomarkers by APOE genotype and gender (*n=126*)

	All			**Female**			**Male**			**Control**	**AD**	***p***	**Control**	**AD**	***p***
	APOE-ϵ4-	**APOE-ϵ4+**	***p***	**APOE-ϵ4-**	**APOE-ϵ4+**	***p***	**APOE-ϵ4-**	**APOE-ϵ4+**	***p***	**APOE-ϵ4-**	**APOE-ϵ4+**		**APOE-ϵ4+**	**APOE-ϵ4-**	
n	71	55		41	32		30	23		45	37		18	26	
Serum B12 (pmol/L)	227 (188, 314)	230 (181, 273)	0.654	236 (194, 227)	251 (194, 311)	0.777	234 (182, 326)	226 (171, 258)	0.322	239 (199, 308)	221 (175, 267)	0.172	252 (74.0, 688)	214 (108, 719)	0.328
Serum folate (nmol/L)	22.0 (14.0, 33.0)	21.0 (13.0, 31.0)	0.511	22.0 (14.0, 37.0)	25.0 (16.2, 44.8)	0.490	21.5 (16.3, 28.8)	15.0 (11.0, 23.0)	0.036	23.0 (19.0, 35.5)	18.0 (12.0, 27.5)	0.035	24.5 (7.00, 45.0)	17.5 (5.00, 45.0)	0.114
RBC folate (nmol/L)	856 ± 338	940 ± 498	0.281	831 ±385	970 ±487	0.177	890 ±262	900 ± 521	0.929	849 ± 282	901 ± 542	0.601	1021 ± 329	866 ± 423	0.227
Homocysteine (µmol/L)	10.4 ±4.30	10.7 ± 3.89	0.671	10.5 ± 5.09	10.1 ± 4.06	0.705	10.1 ± 2.95	11.4 ± 3.59	0.145	9.33 ± 2.17	11.2 ± 3.98	0.012	9.54 ± 3.53	12.2 ± 6.18	0.114
Cysteine (µmol/L)	274 ± 35.9	266 ± 36.3	0.210	276 ± 40.0	271 ± 39.2	0.653	272 ± 30.0	258 ± 31.0	0.111	274 ± 26.9	261 ± 40.8	0.088	277 + 21.8	274 ± 48.3	0.792
Cysteinyl glycine (µmol/L)	24.1 (21.2, 26.9)	23.1 (19.9, 25.4)	0.112	23.3 (21.2, 26.5)	22.8 (19.3, 24.6)	0.070	24.7 (21.7, 27.9)	24.2 (20.6, 27.0)	0.720	24.0 (21.5, 27.1)	21.7 (18.8, 24.8)	0.062	24.3 (15.9, 29.7)	24.6 (16.9, 35.5)	0.886
Glutathione (µmol/L)	10.7 (8.8, 12.6)	10.7 (8.07, 12.5)	0.474	9.62 (8.63, 12.2)	10.8 (8.30, 12.4)	0.689	11.1 (9.18, 13.4)	9.59 (7.25, 12.5)	0.162	10.5 (8.73, 12.3)	10.9 (8.26, 12.4)	0.863	9.83 (5.24, 15.1)	11.0 (5.19, 21.6)	0.115
APOE (µg/mL)	55.5 (43.3, 64.5)	45.6 (38., 67.0)	0.042	57.4 (46.8, 69.9)	46.4 (38.2, 64.7)	0.041	50.1 (41.9, 62.9)	44.2 (37.1, 67.0)	0.419	56.8 (42.7, 65.1)	44.3 (36.4, 69.5)	0.125	46.9 (25.8, 83.5)	53.7 (25.2, 93.0)	0.166
APOE-ϵ4 (µg/mL)		30.3 (17.9, 42.7)			31.0 (17.2, 47.1)			29.7 (21.5, 41.6)							

Notes: Continuous normally distributed variables (RBC folate, homocysteine, and cysteine) are expressed as mean ±standard deviation. Non-normally distributed variables (Serum B12, serum folate, cysteinyl glycine, glutathione, APOE, APOE ϵ4) are displayed as median (interquartile range). Outcomes were considered to be statistically significant at $p < 0.05$. APOE: apolipoprotein E; AD: Alzheimer's disease; RBC: Red blood cell.

Table 5. Thresholds and Predictor Value for likelihood of an AD diagnosis (*n*=126)

	AD			AD/APOE4+		
	Threshold	**AUC**	***p***	**Threshold**	**AUC**	***p***
Biomarker						
Homocysteine (µmol/L)	>11.0	0.629	0.012	NA	NA	>0.05
Cysteine (µmol/L)	<255	0.610	0.033	<253	0.629	0.045
Serum Folate (nmol/L) (All)	<18.0	0.637	0.008	<18.0	0.636	0.035
Serum Folate (nmol/L) (M)	<22.0	0.735	0.003	<22.5	0.819	0.002
Dietary intake						
Total Folate (µg/day) (F)	<336	0.736	0.001	NA	NA	>0.05
Natural Folate (µg/day) (F)	<270	0.687	0.011	NA	NA	>0.05
Riboflavin (mg/day) (F)	<1.12	0.661	0.028	NA	NA	>0.05

Threshold's and *p* values calculated using Receiver Operating Characteristic (ROC) curves. Only significant results are presented (*p*<0.05). Predictor value determined by Area Under the Curve (AUC). Outcomes were considered to be statistically significant at p<0.05. AD: Alzheimer's disease; APOE4: Apolipoprotein E ϵ4; M: Male; F: Female.