Original Paper

Buccal Cytome Biomarkers and Their Association with Plasma Folate, Vitamin B₁₂ and Homocysteine in Alzheimer's Disease

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Key Words

Buccal cytome biomarkers · Folate · Vitamin B₁₂ · Homocysteine · Alzheimer's disease

Abstract

Background/Aims: Alzheimer's disease (AD) is an irreversible neurodegenerative disorder and is the commonest form of dementia. One aim of this study was to determine whether AD individuals have altered plasma folate, vitamin B₁₂ and homocysteine (Hcy) levels compared to controls. The other aim was to investigate correlations between B vitamins and buccal biomarkers to test whether they are influenced by B vitamin status. **Methods:** Folate, vitamin B₁₂ and Hcy were measured using ARCHITECT® and AxSYM® assays. Genomic stability was measured using the buccal micronucleus cytome assay. **Results:** The area under the receiver operating characteristic curve for AD basal cells was 0.96 ($p < 0.0001$), for karyorrhectic cells 0.88 ($p < 0.0001$) and for basal and karyorrhectic cells 0.91 ($p < 0.0001$). Hcy was significantly increased ($p = 0.0003$) compared to controls. Plasma vitamin B₁₂ in controls showed a positive correlation with pyknosis ($r = 0.5365$, $p = 0.004$), karyolysis ($r = 0.5447$, $p = 0.004$) and condensed chromatin ($r = 0.5238$, $p = 0.006$). Plasma vitamin B₁₂ in AD cases showed a positive correlation with micronuclei ($r = 0.3552$, $p = 0.04$) and basal cells ($r = 0.3448$, $p = 0.04$), whilst plasma Hcy showed a negative correlation with karyorrhectic cells ($r = -0.4107$, $p = 0.01$). **Conclusions:** Hcy was significantly increased in AD cases relative to controls. The lower frequency of basal cells and karyorrhectic cells observed in AD cases may be explained by lower vitamin B₁₂ and higher Hcy levels, respectively.

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Introduction

Alzheimer's disease (AD) is a chronic progressive neurological disorder accounting for more than 50% of all clinically diagnosed dementia cases [1]. Studies have shown that AD has been associated with lower levels of folate and vitamin B₁₂ and elevated levels of plasma homocysteine (Hcy) compared to age-matched non-AD controls [2–4]. Hyperhomocysteinemia has been shown to be an important independent risk factor for AD. Hcy not only promotes excitotoxicity, giving rise to neuronal DNA damage and apoptosis, but enhances β -amyloid production through its interaction with presenilins, resulting in the induction of stress proteins such as Herp [5–7]. These findings suggest that Hcy-induced neuronal damage occurring in certain areas of the brain such as the hippocampus, which is involved in short-term memory and learning, may contribute to the cognitive impairment that is characteristic of AD patients [8]. It has recently been shown that a doubling of the total lymphocyte Hcy results in a 36% increase in the likelihood of suffering cognitive impairment later in life [9]. However, the actual concentration of Hcy within the hippocampus of AD patients has yet to be determined.

Folate (vitamin B₉) is an essential B vitamin that is crucial to the prevention of genomic instability and DNA hypomethylation [10, 11]. Folate is required for the synthesis of deoxythymidine monophosphate from deoxyuridine monophosphate, which is essential for DNA synthesis and repair. Under conditions of folate deficiency, deoxyuridine monophosphate accumulates, resulting in the increased incorporation of uracil into DNA leading to single and double strand DNA breaks, chromosome breakage and, ultimately, micronucleus (MN) formation [12, 13]. Folate and vitamin B₁₂ are required in the synthesis of methionine through the remethylation of Hcy and in the synthesis of S-adenosyl methionine (SAM). SAM plays an important role as a methyl donor required for the maintenance of genomic methylation patterns that determine gene expression and DNA conformation, and it is required for the synthesis of myelin, neurotransmitters and membrane phospholipids [14, 15]. Folate deficiency reduces SAM levels, resulting in lower DNA cytosine methylation and elevated levels of Hcy. Additionally, folate deficiency may lead to demethylation of centromeric DNA repeat sequences and centromere dysfunction, resulting in an abnormal chromosome distribution during nuclear division. This, in turn, may result in elevated rates of aneuploidy and altered gene dosage and subsequent gene expression. Folate deficiency has been shown to increase trisomy for chromosome 17 and 21, leading to the possible overexpression of the AD related genes *Tau*, *MPO* and *APP* [16, 17]. Furthermore, in previous studies, we showed that (1) the rate of aneuploidy in buccal cells is increased in AD and (2) the buccal cell cytome is significantly altered in AD cases compared to controls such that the frequencies of basal cells, karyorrhectic cells and condensed chromatin cells are reduced relative to controls, suggesting a diminished regenerative capacity of epithelial tissues [18, 19]. What is unclear is (1) whether single micronutrients or combinations of micronutrients are significantly altered in newly diagnosed AD patients prior to any treatment compared to non-AD individuals and (2) whether nutrient biomarkers are associated with cellular and genomic alterations in peripheral tissues such as the buccal mucosa of healthy older people and those with AD.

The primary aim of this study was to determine whether AD individuals have altered plasma folate, vitamin B₁₂ and Hcy levels compared to age-matched controls. A secondary aim was to investigate correlations between folate, vitamin B₁₂ and Hcy status with previously determined buccal MN cytome assay biomarkers for DNA damage (MN, nuclear buds), cell proliferation (basal, differentiated) and cell death markers (karyolysis; karyorrhectic, pyknotic and condensed chromatin) from these cohorts in order to determine the possibility that the buccal cytome may be influenced by B vitamin status with advancing age.

Table 1. Age, gender ratio and MMSE scores of the study groups

	Controls ¹ (n = 26)	Younger AD group (n = 23)	Older AD group (n = 31)
Mean age ± SD, years	68.7 ± 2.6	70.7 ± 4.1	83.13 ± 4.5
Age range, years	66–75	58–75	78–93
Male/female ratio	11/15	8/15	8/23
Mean MMSE score ± SD	n.a.	22.52 ± 3.013	22.13 ± 3.676
MMSE score range	n.a.	15–27	12–29

n.a. = Not available.

¹ Age and gender matched to the younger AD group.

Subjects and Methods

Recruitment and Characteristics of Participants

Ethics approval for this study was obtained from the Human Research Ethics Committees of the Commonwealth and Scientific Industrial Research Organisation (CSIRO), Food and Nutrition Flagship, the University of Adelaide and the Calvary Hospital Adelaide. Twenty-six healthy controls (age 66–75 years) were recruited through the CSIRO clinical database, and 54 clinically diagnosed AD patients (age 58–93 years) were recruited at the Calvary Private Hospital, Walkerville, S.A., following their initial diagnosis and prior to commencement of any medical treatment. The patients were screened to ensure they were not receiving anti-folate therapy or cancer treatment or having any family history of AD. The initial diagnosis of AD was made by experienced clinicians according to the criteria outlined by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [20], which are the well-recognised standards used in all clinical trials. Following an information session outlining the study, all volunteers consented prior to the sample collection.

The volunteers did not receive any remuneration for their participation in the study. The 54 AD diagnosed individuals were separated into two distinct groups. One group was age matched to the control group and was referred to as the young AD group, whereas the second group was classified as an older AD cohort. Table 1 outlines the demographics of the study cohorts and includes age, gender and the Mini-Mental State Examination (MMSE) scores of the cohorts. There were no significant differences in the gender ratios between the groups (χ^2 test, $p > 0.05$). The age of the control group compared to the younger AD group was not significantly different, but the latter group had a significantly lower age relative to the older AD group ($p < 0.0001$). The MMSE scores of the two AD groups were not significantly different ($p > 0.05$). It should be noted that the MMSE score of the AD cohort ranged from 14 to 29. The MMSE score of 29 at the time of diagnosis was checked following consultation with the diagnosing clinician, revealing that the patient had an atypical disease which was felt to be asymmetrical AD. The patient has been followed up since with ongoing decline. The diagnosis was based on the history, pattern of cognitive deficits including the neuropsychology assessment and brain imaging. The patient had clear atrophy on MRI in the right hippocampal, temporal, parietal and frontal regions together with SPECT perfusion abnormalities consistent with the disease. The pattern of deficits and the background ability resulted in good MMSE scores, but neuropsychological testing showed significant impairment and progressive deficits when repeated 12 months later.

The scores for the control group are not available, but they were 'normal' functioning healthy individuals who self-volunteered and consented to the study and did not report any history of cognitive impairment.

Cell Sampling and Preparation

Blood was collected from the controls by trained nursing staff at the CSIRO Food and Nutrition clinic. From the clinically diagnosed AD patients it was collected prior to medical treatment by trained nursing staff at the College Grove Private Hospital, Walkerville, S.A. Buccal samples were more readily available from the AD cohorts, as visits to the clinic were infrequent and obtaining a fasted blood sample required a second visit within the study time frame. This was not logistically practical, since many individuals lived outside of the city limits and relied on relatives/friends for transportation to the clinic. Consequently, this resulted in more buccal cell samples being made available for analysis as compared to the lower numbers of blood samples.

Blood Collection

A single, 8-ml EDTA tube of fasted blood was collected by venepuncture from all individuals, placed on ice and transported to the CSIRO Genome Health and Personalised Nutrition Laboratory. The blood samples were spun for 20 min at 3,000 rpm (MSE Mistral 2000); 1 ml of plasma was separated for folate and vitamin B₁₂ analysis, and 0.5 ml of plasma for Hcy determination. All samples were snap frozen in liquid nitrogen and stored at –80°C until the analysis could be performed.

Buccal Cell Collection

Buccal cells were collected from all participants by P.T. Cell sampling and collection, the cellular preparation for microscopic analysis and the scoring criteria used for the buccal MN cytome assay have been described previously [21, 22].

Quantification of Plasma Folate, Vitamin B₁₂ and Hcy

Plasma folate was quantified using the ARCHITECT® folate assay (Abbott Laboratories, Abbott Park, Ill., USA), a chemiluminescent microparticle folate-binding protein assay, on the ARCHITECT® i System. Vitamin B₁₂ was quantified using the ARCHITECT® B12 assay (Abbott Laboratories), a chemiluminescent microparticle intrinsic factor assay, on the ARCHITECT® i System. Hcy was quantified using the AxSYM® Hcy assay (Abbott, Wiesbaden, Germany), a fluorescence polarisation immunoassay, on the AxSYM® system. Quantification of folate, vitamin B₁₂ and Hcy was performed by the Division of Clinical Biochemistry at the Institute of Medical and Veterinary Science, Adelaide, S.A.

Statistical Analysis

One-way ANOVA was used to determine the statistical significance of differences in cellular parameters and plasma micronutrient levels measured between the older control, the younger AD and the older AD cohort. Pairwise comparison of significance between these groups was determined using Tukey's test. ANOVA values, positive predictive values, negative predictive values, sensitivity, specificity, likelihood ratios and odds ratios were calculated for basal and karyorrhectic cells using GraphPad PRISM (GraphPad Inc., San Diego, Calif., USA). Receiver operating characteristic (ROC) curves and their area under the curve (AUC) were determined for the cytome biomarkers that were most significantly different in the cohorts. Cross-correlation analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, Ill., USA). Significance was accepted at $p < 0.05$.

Results

Buccal MN Cytome Assay

The significant results for the buccal DNA damage biomarkers (cells with MN or nuclear buds), cell proliferation markers (basal and binucleated cells) and cell death parameters (karyorrhectic, condensed chromatin, karyolytic and pyknotic cells) have been reported elsewhere [19]. The numbers of cells with MN or nuclear buds were slightly elevated in the younger AD group compared to the age-matched controls, although this difference did not achieve significance ($p = 0.12$ and $p = 0.62$, respectively). Basal cells were found to be lower by 81% in the younger AD cohort and by 79% in the older AD cohort compared to the control group ($p < 0.0001$); this was one of the most significant differences found between the cohorts (fig. 1a). The binucleated cell frequency showed no significant difference between the AD and control groups ($p = 0.99$). The frequency of cells with condensed chromatin was found to be reduced by 37.2% in the younger AD cohort and by 56.6% in the older AD cohort when compared to the control group ($p < 0.0001$) (fig. 1b). The frequency of karyorrhectic cells was found to be reduced by 82.9% in the younger AD cohort and by 77.7% in the older AD cohort when compared to the control group ($p < 0.0001$) (fig. 1c). The numbers of pyknotic and karyolytic cells were slightly lower in both AD groups (but not significantly) compared to the controls ($p = 0.47$ and $p = 0.84$, respectively) [19]. The mean and standard deviation values for the various buccal

Fig. 1. Frequency of basal (a), condensed chromatin (b) and karyorrhectic cells (c) in 1,000 buccal cells from controls (n = 30) and younger (n = 23) and older AD groups (n = 31). The plots show the clear separation in frequency of the most different cytochrome biomarkers between the cohorts. Modified and adapted from Thomas et al. [19].

cytochrome biomarkers of the three study groups are presented in table 2. There were no gender differences in biomarkers occurring within each cohort, except for a significant increase in nuclear buds in males compared to females within the young AD group ($p < 0.01$). MMSE scores in the AD cohorts were not significantly correlated with buccal cytochrome assay biomarkers.

Table 2. Values for buccal cytome biomarkers in the study cohorts

	MN frequency	Nuclear bud frequency	Basal cell frequency	Binucleated cell frequency	Condensed chromatin cell frequency	Karyorrhectic cell frequency	Pyknotic cell frequency	Karyolytic cell frequency
Controls	1.12±1.20	1.31±1.38	93.3±43.8 ^a	15.73±9.46	63.62±36.39 ^a	50.73±30.02 ^a	2.04±2.24	114.10±54.58
Younger AD	1.52±1.08	1.65±1.67	17.70±11.38 ^b	15.96±9.32	39.96±28.39 ^b	8.65±11.76 ^b	1.83±1.78	103.50±68.10
Older AD	0.87±1.08	1.25±1.54	19.29±13.98 ^b	15.97±6.79	27.61±15.93 ^b	11.29±12.18 ^b	1.45±1.41	106.70±72.14

Data are presented as means ± SD. Values were derived following the scoring of 1,000 buccal cells from each cohort. Groups not sharing the same superscript letter are significantly different.

Table 3. PPV, NPV, sensitivity, specificity, LR, OR and p values for karyorrhectic and basal cell frequencies

	PPV	NPV	Sensitivity	Specificity	LR	OR (95% CI)	p value
Karyorrhectic cell frequency <30 ¹	85.5%	85.2%	92.1%	74.2%	3.6	33.8 (9.2–124)	0.0001
Basal cell frequency <27 ¹	93.5%	77.8%	84.3%	90.3%	8.7	50.1 (12.3–205)	0.0001
Basal plus karyorrhectic cell frequency <41 ¹	97.7%	76.9%	82.4%	96.8%	25.5	140 (16.8–1,165)	0.0001

Adapted from Thomas et al. [19]. PPV = Positive predictive value; NPV = negative predictive value; LR = likelihood ratio; OR = odds ratio; CI = confidence interval. ¹ Denotes the number of cells in 1,000 buccal cells scored.

Sensitivity, Specificity and ROC Curve Analysis

Values for both basal and karyorrhectic cells were analysed singularly and in combination to determine the sensitivity and specificity of those potential biomarkers which were most different between the control and AD groups. Table 3 lists the sensitivity and specificity data from arbitrary cut-off points for a diagnosis of AD based on basal and karyorrhectic cell frequency. The odds ratio for being diagnosed with AD for a combined karyorrhectic and basal cell frequency value of <40 per 1,000 cells is 140 with a specificity of 97% and a sensitivity of 82%. This would indicate that a false-positive rate for these potential diagnostic biomarkers within the general population would be 2.3% (positive predictive value 97.7%) and that 23.1% (negative predictive value 76.9%) of those individuals tested who are likely to have AD would be falsely detected as normal. The AUC was determined for those cytome biomarkers that were the most significantly different between the cohorts. The AUC for AD basal cell frequency was 0.96 ($p < 0.0001$), for karyorrhectic cells 0.88 ($p < 0.0001$) and for both basal and karyorrhectic cells 0.91 ($p < 0.0001$). The ROC curves for these buccal cytome biomarkers are illustrated in figure 2.

Folate, Vitamin B₁₂ and Hcy

The results for plasma folate, vitamin B₁₂ and Hcy for all cohorts are summarised and illustrated in figure 3. No significant difference was found between the older controls and both AD groups for folate measurements. The older AD group showed a slightly reduced plasma folate level as compared to both older controls and the younger AD group, but this did not reach significance (fig. 3a). All values were within the normal range for plasma folate (6.9–39 nmol/l).

Concentrations of plasma vitamin B₁₂ levels did not show significant differences between the control and AD cohorts ($p = 0.874$) (fig. 3b). All values were within the normal range for plasma vitamin B₁₂ (133–664 pmol/l). It has been shown that MN formation is minimised when vitamin B₁₂ is >300 pmol/l [23]. All subjects achieved this level.

Concentrations of plasma Hcy showed a significant increase in both AD groups compared to the control group ($p = 0.0003$) (fig. 3c). All values were within the normal range for plasma

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Fig. 2. Graphs showing AUC and p values for basal cell frequency (**a**), karyorrhectic cell frequency (**b**) and combined basal and karyorrhectic cell frequency (**c**) for the AD cohort.

Fig. 3. Plasma folate (a), plasma vitamin B₁₂ (b) and plasma total Hcy levels (c) for controls (n = 26) and younger (n = 23) and older AD groups (n = 31). Groups not showing the same letter are significantly different from each other. Error bars denote SE of the mean.

Hcy (5–15 μmol/l). We had previously reported that the MN frequency in lymphocytes of older men and young adults is minimised when Hcy levels are <7.5 μmol/l. All subjects in this study exceeded this level [23, 24].

The results of the cross-correlation analysis between the biomarkers of the buccal MN cytome assay for the controls and for the combined AD cohorts and plasma folate, vitamin B₁₂ and Hcy levels are shown in tables 4 and 5. For the older controls, plasma folate and Hcy showed no significant correlation with any of the buccal cytome parameters measured. Plasma vitamin B₁₂ was positively correlated with pyknosis ($r = 0.5365$, $p = 0.0047$), kary-

Table 4. Cross-correlation results between biomarkers of the buccal MN cytome assay and plasma micronutrients for the controls (n = 30)

		Plasma folate	Plasma vitamin B ₁₂	Plasma Hcy
Number of buccal cells with MN	Pearson correlation	–0.0234	–0.0880	–0.1665
	significance (2-tailed)	0.9095	0.6688	0.4163
Number of MN in 1,000 cells	Pearson correlation	–0.1828	–0.1041	0.0183
	significance (2-tailed)	0.3714	0.6128	0.9292
Karyorrhexis	Pearson correlation	0.1414	0.0948	–0.3020
	significance (2-tailed)	0.4907	0.6449	0.1337
Pyknosis	Pearson correlation	–0.0330	0.5365	–0.2053
	significance (2-tailed)	0.8727	0.0047**	0.3144
Binucleated cells	Pearson correlation	–0.0887	0.0715	–0.0958
	significance (2-tailed)	0.6663	0.7284	0.6413
Karyolysis	Pearson correlation	0.2308	0.5447	–0.0422
	significance (2-tailed)	0.2566	0.0040**	0.8376
Nuclear buds	Pearson correlation	–0.2463	0.1320	–0.2221
	significance (2-tailed)	0.2251	0.5202	0.2754
Condensed chromatin	Pearson correlation	0.1212	0.5238	–0.2808
	significance (2-tailed)	0.5554	0.0060**	0.1646
Basal cells	Pearson correlation	–0.0390	–0.0113	0.1106
	significance (2-tailed)	0.8500	0.9559	0.5906
** Correlation significant at the 0.01 level (2-tailed).				

olysis ($r = 0.5447$, $p = 0.0040$) and condensed chromatin ($r = 0.5238$, $p = 0.0060$) (table 4). In the AD cohort, plasma folate showed no correlation with any of the buccal parameters measured. Plasma vitamin B₁₂ showed a positive correlation with the number of MN scored in 1,000 cells ($r = 0.3552$, $p = 0.0425$) and with the number of basal cells ($r = 0.3448$, $p = 0.0494$) scored in the assay. Plasma Hcy showed a negative correlation with the number of karyorrhectic cells ($r = -0.4107$, $p = 0.0176$) scored in the assay (table 5). No significant correlation was found between MMSE scores of AD subjects and plasma folate, vitamin B₁₂ and Hcy.

Discussion

The aims of this pilot study were to investigate levels of plasma folate, vitamin B₁₂ and Hcy in AD patients compared to age and gender matched healthy controls and to investigate their correlation with buccal cytome biomarkers associated with AD. It has previously been shown that some AD patients have reduced levels of folate, making them susceptible to genomic instability events [25–27]. In this study, only a slight non-significant reduction in plasma folate levels was found for the AD cohorts compared to the age matched older controls. However, it is possible that although plasma levels appear to be adequate, bioavailability of folate may still be impaired, as many physiological and biochemical processes are required to effectively deliver dietary folate from plasma into the cells [28]. Furthermore, defects in the uptake and storage of folate could result in an apparent increase in plasma folate [29,

Table 5. Cross-correlation results between biomarkers of the buccal MN cytome assay and plasma micronutrients for the AD cohort (n = 54)

		Plasma folate	Plasma vitamin B ₁₂	Plasma Hcy
Number of buccal cells with MN	Pearson correlation	–0.0975	0.2120	–0.1189
	significance (2-tailed)	0.5893	0.2363	0.5098
Number of MN in 1,000 cells	Pearson correlation	–0.2361	0.3552	–0.0963
	significance (2-tailed)	0.1860	0.0425*	0.5938
Karyorrhexis	Pearson correlation	0.0810	0.2808	–0.4107
	significance (2-tailed)	0.6537	0.1134	0.0176*
Pyknosis	Pearson correlation	0.0171	0.0918	–0.2431
	significance (2-tailed)	0.9244	0.6112	0.1728
Binucleated cells	Pearson correlation	0.1507	–0.1013	0.1382
	significance (2-tailed)	0.4025	0.5749	0.4433
Karyolysis	Pearson correlation	–0.1847	–0.0181	0.0419
	significance (2-tailed)	0.3036	0.9203	0.8165
Nuclear buds	Pearson correlation	0.2468	0.1977	–0.3142
	significance (2-tailed)	0.1733	0.2781	0.0799
Condensed chromatin	Pearson correlation	0.0553	–0.1477	0.1110
	significance (2-tailed)	0.7596	0.4122	0.5385
Basal cells	Pearson correlation	–0.0201	0.3448	–0.1473
	significance (2-tailed)	0.9113	0.0494*	0.4133
MMSE score	Pearson correlation	0.2359	0.0035	–0.0664
	significance (2-tailed)	0.1862	0.9844	0.7134

* Correlation significant at the 0.05 level (2-tailed).

30]. It may be that measurement of red blood cell folate would give a more accurate determination of long-term folate status and should be considered for future studies.

Plasma levels for vitamin B₁₂ showed no significant differences between any of the cohorts investigated. Vitamin B₁₂ is an essential cofactor for methionine synthase, but in order to be biologically effective it needs to be in a reduced state. AD has been shown to be associated with oxidative stress [31–33]. It is possible that under such conditions oxidative stress could lead to deactivation of cob(I)alamin by its subsequent oxidation to cob(II)alamin, resulting in a functional vitamin B₁₂ deficiency [34]. This in turn could lead to reduced methionine synthase activity, resulting in Hcy accumulation following failure to be converted to methionine together with an inability to convert 5-methyltetrahydrofolate to tetrahydrofolate, the form of folate that is polyglutamated and stored [35, 36]. Such changes in the oxidation state were not determined in the current analysis but would be of great interest in any future studies undertaken, given the evidence for increased oxidative stress in AD [37–40]. Alternatively, as with folate, plasma vitamin B₁₂ levels do not reflect the bioavailability of vitamin B₁₂ at the cellular level. Physiologically, in order to deliver vitamin B₁₂ from the gut to the tissues, at least five peptides are necessary (R binder, intrinsic factor, ileal receptors and transcobalamin I and II), and a further four enzymes are required to obtain the reduced state for effective methionine synthase function (cbIF, cbIC/D, cbIE/G and microsomal reductase) [41]. It is possible that any dysfunctionality in these proteins may lead to a func-

tional vitamin B₁₂ deficiency which would not be reflected in a plasma vitamin B₁₂ analysis. Transcobalamin II and methylmalonyl-CoA are considered to be better biomarkers of functional vitamin B₁₂ status and should be incorporated into future studies [42, 43].

Plasma Hcy was found to be significantly increased in both AD cohorts compared to the age matched controls, confirming the results of earlier investigations [44–46]. There is substantial evidence to show that total Hcy is an independent vascular risk factor, and individuals with such risk factors and cerebrovascular disease have been shown to have an increased risk of developing AD [47, 48]. Hyperhomocysteinaemia sensitises nervous tissue to increased glutamate toxicity via activation of N-methyl-D-aspartate receptors and damages neuronal DNA, giving rise to apoptosis [5]. There is also evidence that elevated levels of Hcy are associated with increased β -amyloid peptide generation, impairment of DNA repair and sensitisation of neurons to amyloid toxicity [5, 49]. It has been proposed that such events result in neuronal damage within the hippocampus, leading to cognitive impairment which is characteristic of AD.

Finally, a cross-correlation analysis was performed between the biomarkers of the buccal MN cytome assay and levels of folate, vitamin B₁₂ and Hcy for the older and combined AD cohorts. The analysis showed that the results from the control cohort showed a positive correlation for vitamin B₁₂ with the cell death parameters pyknosis, karyolysis and condensed chromatin, but no significant correlation was evident for either folate or Hcy (table 4). These results suggest that vitamin B₁₂ may in some way facilitate the cell death process in normal buccal mucosa, and they could explain the association of vitamin B₁₂ with lower MN frequency observed in previous studies [50]. However, no association with MN frequency was observed in this study. On the other hand, vitamin B₁₂ deficiency tends to reduce the regenerative capacity of tissues and promotes apoptosis [51, 52]. An alternative explanation is that vitamin B₁₂ promotes the turnover of the buccal mucosa and the elimination of excess cells by normal cell death processes intended to maintain a normal oral mucosa thickness [53].

In the AD cohort, folate showed no significant correlation with any of the buccal parameters measured. Plasma vitamin B₁₂ showed a significant positive correlation with the number of basal cells ($r = 0.3448$, $p = 0.04$), demonstrating an association with the proliferative index of these cells suggesting an increased regenerative capacity. Plasma vitamin B₁₂ also showed a slight positive correlation with the number of MN scored ($r = 0.3552$, $p = 0.04$), which may be explained by the fact that MN are mainly expressed in dividing cells which excess vitamin B₁₂ or the foods it is derived from (e.g. meat proteins, which are a rich source of methionine) tend to promote [54]. Further dose response experiments involving vitamin B₁₂ are necessary in order to determine the relationship between vitamin B₁₂ and these cytome measures. Plasma Hcy showed a negative correlation with the number of karyorrhectic cells ($r = -0.4107$, $p = 0.017$), indicating a suppressive effect on cell death under conditions of high Hcy levels. In the AD cohort, plasma Hcy was elevated and the number of karyorrhectic cells scored in the buccal cytome assay was significantly reduced compared to age matched controls with a lower Hcy level, suggesting a consistency of Hcy and karyorrhexis as risk markers for AD that may be connected metabolically.

Conclusions

To the authors' knowledge, this is the first time that associations between micronutrient status and changes in buccal cytome biomarkers have been made in relation to AD status. Plasma Hcy was significantly increased in AD cases relative to controls. The lower frequency of both basal and karyorrhectic cells observed in the AD cohort may be explained by lower vitamin B₁₂ and higher Hcy levels, respectively.

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Disclosure Statement

The authors report no conflict of interest in relation to this study.

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