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Peter D Fransquet, Karen Ritchie, Vania Januar, Richard Saffery, Marie-Laure Ancelin, Joanne Ryan

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Is peripheral *BDNF* promoter methylation a preclinical biomarker of dementia?

Peter D. Fransquet[1,2], Karen Ritchie[3,4], Vania Januar[2], Richard Saffery[2], Marie-Laure Ancelin[3], *Joanne Ryan[1,2,3]

[1]Biological Neuropsychiatry and Dementia Unit, School of Public Health and Preventive Medicine, Monash University, Melbourne, Victoria, Australia;

[2]Epigenetics Group, Murdoch Childrens Research Institute, Royal Children's Hospital, University of Melbourne, Parkville, Victoria, Australia;

[3]Inserm U1061, Hospital La Colombiere & University Montpellier, Montpellier F-34093, France.

[4]Center for Clinical Brain Sciences, University of Edinburgh, Edinburgh, United Kingdom

Running title: Blood *BDNF* methylation as dementia biomarker

*Corresponding author: School of Public Health and Preventive Medicine, Monash University, Melbourne, Victoria, Australia. T: +61 3 9903 0200, E:joanne.ryan@monash.edu

ABSTRACT

Brain derived neurotrophic factor (BDNF) has been implicated in dementia. Preliminary evidence suggests that *BDNF* DNA methylation may be a diagnostic biomarker of dementia, but the potential pre-clinical utility remains unclear. Participants in the ESPRIT study were assessed for cognitive function and dementia (DSM-IV criteria) over 14-years. *BDNF* exon 1 promoter methylation was measured in blood at baseline (n=769), and buccal samples during follow-up (n=1062). Genotyping was carried out for several common *BDNF* SNPs, including Val66Met (*rs6265)* and *APOE-ε4.* Multivariable logistic regression analyses determined the association between *BDNF* methylation and both prevalent and incident dementia. Adjustment for gender, age, education, *APOE-ε4* genotype, body mass index, depression, and type 2 diabetes, as well as possible effect modification by gender and genetic variation were also investigated.

Weak evidence of an association between lower blood methylation and dementia was observed at one of 11 sites investigated (Δ-0.5%, 95%CI:-0.9,-0.04, p=0.03, p=0.22 adjusted for multiple comparisons). Buccal methylation at two other sites was associated with 14-year incident dementia cases prior to adjustment for multiple comparisons only, and the effect sizes were small (Δ+0.3%, OR:1.57, SE:0.30, p=0.02, p=0.14 adjusted and Δ-1.5%, OR:0.85, SE:0.06, p=0.03, p=0.14 adjusted). Genetic variation in the *BDNF* gene did not modify these associations, and no gender-specific effects were observed. There was only a weak correlation between blood and buccal *BDNF* log-methylation at two sites (both r=-0.11). There was no strong evidence that blood or buccal *BDNF* exon 1 promoter DNA methylation is associated with prevalent or incident dementia, and reported associations would not remain after adjustment for multiple testing.

Keywords: *BDNF*; blood; DNA methylation; epigenetics; dementia; biomarkers

INTRODUCTION

Dementia is the umbrella term used to describe a group of symptoms that results in long-term decline of cognitive functioning, which is significant enough to affect daily life [1]. It is a prevalent, debilitating condition and one of the leading contributors to the overall burden of disease, with significant impacts on the individual, family and society [2]. Over 50 million people worldwide are thought to have dementia, and dementia prevalence is projected to rise to >150 million by 2050 [3]. Further, it is a multi-factorial disease with complex aetiology that is yet to be completely defined [4].

There is strong interest in identifying robust biomarkers for dementia which would assist in accurate and timely diagnosis. A biomarker which could be readily analysed from a blood sample or buccal swab would enable a relatively easy and non-invasive, cost effective screening [5, 6]. As dementia pathophysiology manifests many years prior to symptom onset, a preclinical biomarker would hold greatest utility, with the future possibility for early intervention [7].

Epigenetic biomarkers are a promising new class of measures with a growing body of evidence indicating their potential clinical utility [8]. Epigenetics, refers to dynamic modifications to the genome, with the potential to impact its activity, in response to environmental stimuli or genetic factors [9]. The most well studied epigenetic mechanism is DNA methylation, wherein a methyl group is covalently attached to cytosine of cytosine-phosphate-guanine (CpG) base pairs within DNA [10]. DNA methylation is largely conserved across tissues, although there are key sites of differential methylation associated with tissue specificity [11].

Recent research suggests the disruption of epigenetic processes in the brain in individuals with dementia [12]. Correlations have been made between blood and brain methylation at certain CpG sites, suggesting that methylation measures in blood have the potential to represent methylation levels in the brain [13]. Buccal epithelial cells are also a possible surrogate for

neural tissues in methylation studies, as both tissues are derived from embryonic ectoderm during development [14]. Although epigenetic biomarker research in dementia is still in its infancy, there is some preliminary data to suggest that differently methylated genes can be found in the blood of demented patients compared to controls [15].

Brain-derived neurotrophic factor (BDNF) is a neurotrophin protein, important in the regulation of neuronal activity and neurogenesis [16]. BDNF is thought to be neuroprotective against amyloid beta toxicity, one of the primary hallmarks of Alzheimer's disease (AD), the most common form of dementia [17, 18]. As well, lower serum BDNF levels have been reported in individuals with dementia [19].

Our recent systematic review of the utility of blood DNA methylation as a biomarker for dementia, suggested that methylation of *BDNF* was one of the more promising candidates [15]. Two small case-control studies (n=106 and 18) reported higher *BDNF* DNA methylation in dementia cases compared to controls [20, 21]. A third study (n=39) however, found no association [22]. Importantly, all three studies investigated DNA methylation in individuals with dementia, so they could not conclude whether these marks were present prior to diagnosis, and thus their potential as pre-symptomatic biomarkers is unknown.

An important consideration when looking at DNA methylation is underlying genetic variation, [23, 24], but none of the previous case-control studies have considered this. *BDNF* single nucleotide polymorphism (SNP) *rs6265*, also known as Val66Met, has been associated with dementia neuropathophysiology, particularly in AD [25] and cognitive impairment [26]. In addition to variation in the *BDNF* gene, *APOE* is the single biggest genetic risk factor for late-onset dementia [27, 28]. It is possible that genetic variation in the *APOE* gene can also interact to modify the association between *BDNF* DNA methylation and dementia.

The primary aim of this study was to determine whether blood DNA methylation levels at *BDNF* were associated with the prevalence and incidence of dementia in a community-based population. Secondary aims were to ascertain whether underlying genetic variation influences these associations, and to investigate the correlation between blood and buccal *BDNF* methylation.

METHODS

ESPRIT cohort

This study included participants from the Enquête de Santé Psychologique–Risques, Incidence et Traitement (ESPRIT) cohort, a longitudinal study of psychiatric disorders in an aging French population [29]. To be included in ESPRIT, participants needed to be over 65 years old, not institutionalised, and residing in the Montpellier region. They were predominantly (>99%) of white European ancestry [30]. Participants answered standardized questionnaires, and underwent extensive clinical assessments at their inclusion, and at up to seven follow-up waves (with approximately 2-3 years between each). Written and informed consent was given by each participant, and ethical approval was granted by the regional ethics committee (Ethical Committee of Sud Méditerranée III and University Hospital of Kremlin-Bicêtre, France, project number DGS2007-0477).

Assessment of cognitive function

Cognitive tests were administered by trained staff at baseline and each follow-up interview. These included global cognition (Mini-Mental State Examination (MMSE) [31]), visual memory (Benton's Visual Retention Test [32]), verbal fluency (Isaacs Set Test [33]), psychomotor speed (Trail Making Test, Part A [34]), and executive function (Trail Making Test, Part B [34]).

Diagnosis of dementia

Dementia status was assessed over a 14-year period. A preliminary diagnosis and classification of dementia at each follow-up examination was made by clinical study investigators according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) revised criteria [35, 36]. These were further validated by a national panel of neurologists, independent of the ESPRIT investigators. The date of dementia onset was the date of the follow-up interview when dementia was diagnosed. All-cause dementia was the primary outcome in this study, but probable and possible Alzheimer's disease were also considered.

Other characteristics

Education was classed as having completed at least secondary school education or not. Mini-Mental State Examination, as a measure of global cognitive function, was considered as normal (>26) or low (<=26). Smokers were classed as ever smoking (>1 pack year), versus not smoking (<1 pack year). Alcohol use was categorized as either high (>= 24grams per day) or low (<= 24grams per day). Participants were categorized as obese if body mass index (BMI) was => 30, and diabetes was recorded as high glucose levels or treatment. Depression was defined as detailed previously [37]. Past or current depressive disorders were diagnosed by trained professionals according to DSM-IV criteria. The Center for Epidemiologic Studies Depression (CES-D) scale was used to assess depressive symptoms [38]. Participants were categorised as having depression if they had a current diagnosis of major depression or a CES-D score ≥16, which is representative of moderate to severe depression.

Methylation analysis for BDNF exon I promoter

Methylation data were generated using blood samples from 769 participants collected at baseline, and buccal samples for 1062 collected at approximately the fourth wave (7 year)

follow-up. Genomic DNA was extracted from white blood cells in 15ml EDTA using Amersham-Pharmacia Biotechusing DNA extraction kits [39]. Four-hundred nanograms of extracted DNA was used for bisulphite conversion (EZ-96 DNA Methylation-Lightning MagPrep kit, Irvine, USA). Genomic DNA was extracted from buccal swabs by salting out, and bi-sulphite converted using methods described above. DNA was subsequently amplified in triplicate from both tissues.

An assay targeting a CpG island in *BDNF* exon I promoter region was designed using Agena Bioscience Epidesigner (epidesigner.com), covering 254 base pairs (chr11: 27,744,025 - 27,744,278, hg19). The amplification primers were:

forward: 5'-aggaagagagGGTAGAGGTAGGGAGATTTTATGTTAG-3'

reverse: 5'-cagtaatacgactcactatagggagaaggctCCTACCCCCACTCTAATTAAAACAA-3'.

The Sequenom MassARRAY EpiTYPER platform was used to obtain DNA methylation data for 11 analytical CpG units (**Table S1**) [40]. These units comprised 16 individual CpG sites, with some units having multiple CpG sites. In the case of a single methylation measure (unit) comprising multiple individual sites, the average methylation was given. The average methylation level for samples with technical replicate values within 10% of the median were included in analysis. Three-hundred and eighty-three participants with analysable blood methylation data had matching buccal methylation data. To normalise both blood and buccal methylation data, values were log-transformed for analysis. Where raw methylation values were '0', data were replaced with '0.0001' to avoid undefined log-transformed results. Where evidence of association was seen between log-methylation and any variable, difference in mean untransformed data is reported.

BDNF and APOE genotyping

Genotyping was performed for seven polymorphisms across the *BDNF* gene region, including *rs6265, rs11030101, rs28722151, rs7103411, rs962369, rs908867* and *rs1491850*. These were chosen as they have previously been implicated in neurological disorders [37]. *BDNF* genotyping was carried out using buccal DNA, using the KBioscience Competitive Allele-Specific PCR SNP genotyping system (KASPar) and performed by KBiosciences [41]. *APOE* genotyping was carried out at Lille Genopole in Northern France (http://www.genopole-lille.fr/spip/) using DNA extracted from fasting blood samples (Puregene Kit, Qiagen, France) [42].

BDNF genotype frequencies can be seen in **Table S2**. Binary variables were generated, grouped as either homozygous for major allele, or heterozygous/homozygous for minor allele (**Table S3**). For example, *rs6265* genotypes were grouped as homozygous major (Val/Val (GG)) or heterozygous (Val/Met (GA)) plus homozygous minor (Met/Met (AA)). In the case of *APOE*, it was grouped as at least one ε4 allele (cytosine at either *rs429358* or *rs7412*), versus none (**Tables 1 and S4**).

Statistical analysis

T-tests were used to determine the unadjusted association between blood log-methylation and prevalent dementia at baseline, incident dementia (over 14-year period) and all dementia cases (at baseline or over follow-up). Cohen's *d* was used to calculate the effect size. Logistic regression analysis was used to determine the association between blood log-methylation at baseline and dementia, accounting for a number of possible confounding factors. This included factors which are known to be associated with dementia risk and could possibly be associated with variations in DNA methylation levels, for example age, gender (women have increased risk of dementia [43], and there are clear DNA methylation differences between men and women [44]), *APOE-ε4* genotype, education level [45], BMI [46], depression [47], and diabetes

[48]. Interactions were also assessed between *BDNF* methylation and *APOE-ε4* status, gender, and *rs6265*, however no evidence of effect modification was found

To determine whether the findings were consistent across tissues, logistic regression analysis was also used to investigate the association between buccal DNA methylation and incident dementia. Correlation coefficients were used to determine the extent to which there was a linear correlation between blood and buccal *BDNF* methylation levels. Equivalence testing was carried out using two one-sided tests (TOST) with the R Package "TOSTER" [49] to provide further support for null findings. Adjustment for multiple comparisons was carried out using the Benjamin-Hochberg (BH) procedure, and adjusted BH p-values reported for each test [50, 51].

RESULTS

Study population

Characteristics of the participants included in the study are shown in **Table 1**. Individuals with prevalent dementia were older than individuals without dementia. Older age was also predictive of 14-year incident dementia. Lower MMSE was associated with both prevalent and incidence dementia, as was the *APOE-ε4* allele. Depression and diabetes also differed between individuals with incident (but not prevalent) dementia and individuals without. No other characteristics differed significantly between groups.

The initial analysis of prevalent dementia includes all 769 participants with blood samples (n = 20 prevalent dementia, n = 749 without dementia). The subsequent analysis of incident dementia involves only 749 participants, as the 20 individuals with prevalent dementia were excluded.

Unadjusted associations between blood BDNF DNA methylation and dementia

There was no strong evidence for an association between *BDNF* methylation and dementia prevalence at any CpG unit (**Figure S1**). The only small differences that were observed was slightly lower *BDNF* methylation in individuals with dementia, compared to those without at CpG_6 (T-test: Δ -0.9%, 95%CI:-1.8,0.9, p=0.08, TOST: 90%CI:-0.016,-0.001, p<0.001), and CpG_7_8_9 (T-test: Δ -0.5%, 95%CI:-1.1,0.04, p=0.07, TOST: 90%CI:-0.01,0, p<0.001), however these did not reach significance. Over the 14-year follow-up period, 70 participants were diagnosed with dementia, and 679 remained without dementia. No evidence was observed of an association between *BDNF* DNA methylation and incident dementia **(Figure 1**). When combining both prevalent and incidence cases (**Figure S2**), slightly lower baseline *BDNF* methylation at CpG_6 (Δ -0.5%, 95%CI:-0.9,-0.04, p=0.03, p=0.22 BH adjusted, Cohen's *d*: 0.24) was observed in individuals with dementia, compared to those without dementia. When considering individuals diagnosed with AD specifically (n=54) prevalent and incidence cases of total dementia), results remained very similar (e.g. CpG_6 (Δ -0.6%, 95%CI:-1.2,-0.05, p=0.03, p=0.37 BH adjusted, Cohen's *d*: 0.30). Neither observation passed adjustment for multiple comparisons.

BDNF methylation and genetic variation

There was no association between genetic variation in *APOE-ε4*, *rs6265, rs1491850, rs28722151 and rs7103411* and *BDNF* methylation (data not shown). Before BH adjustment, an association was seen between *rs692369* and lower methylation at CpG_10 (Δ -1.3%, 95%CI:-0.06,-2.5, p=0.04, p=0.38 BH adjusted), *rs11030101* and higher methylation at CpG_25 (Δ +0.6%, 95%CI:0.07,1.1, p=0.03, p=0.27 BH adjusted), and *rs908867* and higher methylation at CpG_6 (Δ +0.5%, 95%CI:0.09,0.9, p=0.02, p=0.20 BH adjusted). None of the

other *BDNF* genetic variants were associated with either prevalent or incidence dementia (**Table S3**).

Regression analysis

As there was some evidence that *BDNF* methylation at CpG_6 and CpG_7_8_9 may be associated with dementia, logistic regression models were generated with adjustment for potential confounding factors. The addition of gender, age, education and *APOE*-ε4 genotype had little influence on the results (**Table 2**), nor did the addition of obesity, depression and diabetes on CpG_6 (OR: 0.74, SE: 0.10, p=0.02, p=0.23 BH adjusted), but it did for CpG_7_8_9 (OR: 0.85, SE: 0.11, p=0.21, p=0.61 BH adjusted).

Buccal BDNF DNA methylation and incident dementia

Buccal methylation data was available from 1062 participants (n=97 incident dementia cases, 965 without dementia). Characteristics of participants that provided buccal samples are shown in **Table S4.** Age, *APOE-ε4* genotype, MMSE, BMI, and diabetes differed significantly between controls and the incident dementia group. To determine whether findings in blood could be replicated in buccal tissue, logistic regression was performed (**Table S5**). We found no evidence of an association between CpG_6 methylation and dementia status (**Figure 2**). However, higher methylation at CpG_7_8_9 was associated with incident dementia (Δ +0.3%, 95%CI:0.02,0.5, p=0.04, Cohen's *d*: -0.22), even after adjusting for confounding factors (Model 2), including sex, age, education, *APOE-ε4* genotype, obesity, and diabetes (OR: 1.57, SE: 0.30, p=0.02). DNA methylation at CpG_14 was also associated with incident dementia using log transformed data (Δ -0.43%, 95%CI:-0.10,-0.75, p=0.0097, Cohen's d 0.30) and remained after adjustments for confounding factors (Model 2, OR: 0.85, SE: 0.06, p=0.03). After adjustment for multiple comparisons however, none of the associations remained significant.

Correlation between blood and buccal BDNF DNA methylation

Of the participants included in this study, 383 participants had methylation data for both blood and buccal tissues. This included 35 individuals with incident dementia and 348 who were without dementia (**Table S6**). Using log-methylation values, CpG_6 (n=378, r = -0.11) and CpG_15 (n=377, r = -0.11) showed a weak negative correlation between blood and buccal methylation (**Figure S3**).

DISCUSSION

We found there is no strong evidence that blood methylation of *BDNF* has the potential to be a biomarker for preclinical or diagnosed dementia. Differential methylation at two CpG's were associated with prevalent dementia, one of which was also associated with incident, and prevalent plus incident dementia. A higher buccal methylation at one CpG was associated with incident dementia, even after adjusting for multiple covariates. However, the effects were small, and equivalence testing indicated that null observations were statistically equivalent to zero. Furthermore, these associations would not remain after adjustment for multiple comparisons. Genetic variation across the *BDNF* region was only weakly associated with three specific *BDNF* methylation sites, but in these instances, did not modify the association between methylation and dementia status. Likewise, consideration of *APOE-ε4* genotype or potential gender-specific associations did not modify the findings.

There have been only three previous case-control studies which have measured *BDNF* methylation and investigated its association with prevalent dementia; they were limited in size and did not consider confounding factors. One of these studies included 44 individuals with AD (mean age 80, SD 8.92) and 62 control individuals (mean age 79.63, SD 7.85) all of whom were recruited from hospitals in China [20]. AD was diagnosed using ICD-10 criteria [52], as well as a medical history, brain imaging, and cognitive screening tests including MMSE. Controls

were free of any neurological disorders, although exact assessments undertaken are not clear. The methylation of four CpG units of *BDNF* exon I promoter region, 120 bases downstream from the region within our assay, was measured in peripheral blood using pyrosequencing. All four CpG's were found to be higher in the AD group compared to controls, as was average methylation over these four sites (AD 9.50% ± 4.43, CT 7.45% ± 2.77, p=0.004). When stratified by sex, only one of the four sites remained significant in males, while another was significant in females. However, the number of males or females were not stated. It appears that no adjustment was made for potential confounding factors.

Another study included 6 controls and 12 AD cases (mean age 66.5, SD 5.07, 60% women) consisting of outpatients from a memory clinic and a University hospital in Japan [21]. Probable AD was diagnosed using National Institute of Neurology and Communicative Disorder and Stroke/Alzheimer Disease and Related Disorder Association (NINCDS/ADRDA) criteria, as well as a behavioural test (behave-AD) and cognitive tests including MMSE and the Frontal Assessment Battery [53-55]. No cognitive assessment of controls was reported, however those with a self-reported history of any psychiatric disorder were excluded. In this study they also measured blood *BDNF* methylation at exon I promoter region (covering 20 CpG sites in a different CpG island), using a slightly different method (ABI 3730 DNA analyser) approximately 21,000 base pairs downstream from the region targeted in our study. In a similar manner to the previous study, they found total methylation ratio (including all 20 CpG's) to be significantly higher in the AD group compared to controls (5.08 ± 5.52% vs 2.09 ± 0.81%; p=0.04). One particular site was much higher in the AD group compared to controls (4.85% ± 6.91, 0.00% ± 0.00, p=0.01), although only univariate analysis was presented.

While these two studies conflict with the findings from our analysis, involving a larger cohort of population-based individuals, a third case-control reported findings that support those of our work. This study involving Caucasian Italian men (20 AD, 19 controls; mean age 75 (SD 7),

found no association between blood *BDNF* methylation and AD status [22]. AD was diagnosed using the DSM-III-R and NINCDS/ADRDA criteria, including cognitive assessments using MMSE. It was unclear if control participants were cognitively assessed. Methylation was measured in circulating leukocytes (using methylation-specific RT-PCR). It is unclear which region of the *BDNF* gene was investigated, and they did not state the number of CpG sites analysed.

In addition to these case-control studies which specifically focused on *BDNF*, there have now been a number of epigenome-wide associated DNA methylation studies of dementia, predominantly using blood [15, 56]. None of these studies identified the *BDNF* gene or nearby region, as one of the significant findings. This aligns with our current study, where there was no strong evidence in adjusted analysis, for an association between blood *BDNF* methylation and both prevalent and incident dementia.

The contrasting findings of previous studies could be due to a number of reasons. As part of our previously mentioned systematic review, we assessed the quality of evidence in all included studies, using Joanna Briggs Institute criteria [57]. The three aforementioned studies scored quite low (44.4% - 66.7%) in critical appraisal. The primary reason for this was that these studies failed to identify, or adjust for possible confounding factors. The two studies to find associations between *BDNF* blood methylation and dementia were small, and potentially underpowered to find such large methylation differences, suggesting the possibility of false positive results. Given this, the strength of evidence within these studies is weak, and could thus be the cause of contradictory results. Based on our sample size and the number of prevalent and incident dementia cases, our study had over >80% power (at 5% significance level) to detect relatively small effect sizes in blood of ≥1.3% and 0.64% (SD 2) for prevalent and incident dementia respectively, and in buccal of ≥0.61% (SD 2) for incident dementia. Thus, our study was adequately powered to observe previously reported effect sizes (Δ 2.99% and Δ

5.07% respectively) [20, 21]. However, as all studies examined slightly different genomic regions, it is hard to make direct comparisons. Finally, previous studies included different ethnic groups (Chinese, Japanese, Italian, French). As it is well recognised that there are ethnic-specific genetic and epigenetic patterns, as well as associations with health outcomes, this must be considered as one possible reason for the discrepancy in findings between studies (e.g. in Asian versus Caucasian populations) [58, 59].

An advantage of our study over previous studies was the opportunity to assess blood-based methylation as a preclinical biomarker of incident dementia. All previous studies have focused on prevalent AD only. Differences between our study and previous studies are unlikely to be due to the analysis of all-cause dementia versus AD. Indeed, when we looked specifically at AD, the findings remained similar. Another strength of our study was the consideration of genetic variation, in particular *APOE-ε4* and *rs6265* genotypes, which are of particular interest for dementia. There was no evidence that genetic variation influenced or modified the association between *BDNF* DNA methylation and dementia.

While it has been common to use both blood and buccal tissues in some methylation studies, to our knowledge this is the first study to investigate correlations between inter-individual blood and buccal tissue *BDNF* methylation. Only very weak correlations were found between blood and buccal tissues at two CpGs, and only CpG_15 remained associated when looking at raw methylation values. Neither passed adjustment for multiple comparisons. It should be noted however, that the biological samples were not taken at the same time, and given that DNA methylation can change over time, this could partly account for the weak correlations across tissue [60, 61]. The inverse correlations were somewhat surprising, however DNA methylation is known to vary across tissues [11]. A previous study has explored the inter-individual correlation between methylation of brain and peripheral tissues such as blood, buccal, and saliva [62]. They reported that the proportion of correlated CpGs within *BDNF* between brain and

peripheral tissues is low (6 to 18%). Similar to our study, they also found relatively weak correlations (r = 0.15 to 0.19), however this was between brain and peripheral tissues, rather than between blood and buccal tissue.

Future directions

As dementia is a progressive disease, and DNA methylation changes over time, future studies should consider looking at measures of mild cognitive impairment (MCI), which can be considered to precede dementia [63]. Two studies by the same group measured *BDNF* methylation using pyrosequencing (3 CpGs in promoter I and 4 CpGs promoter IV) in peripheral blood of controls (n=728, mean age 66.9, SD:6.32) and participants with amnestic MCI (n=506, mean age 67.2, SD:6.95) [64, 65]. They also followed the 506 individuals with MCI over five years and compared methylation between those that remained cognitively stable (n=330, mean age 69.9, SD:4.01), and those that converted to AD (n=128, mean age 76.0, SD:4.82). These studies found that methylation was associated with MCI vs controls, higher at two CpGs in each promoter, as well as average methylation (MCI: 16.11% ± 4.48, Controls: 14.85% ± 4.07, $p < 0.001$). The methylation of these same four CpGs was found to be significantly higher in AD converted from MCI, compared to stable MCI, as well as average methylation (AD: 19.92% ± 5.89 versus MCI: 17.43% ± 5.10, $p < 0.001$). Results were adjusted for age, education, and gender. Cognitive scores were also added in multivariate Cox regression, although it is not clear which measure, or if multiple were included. In these models they found one CpG in promoter IV to be associated with AD conversion from MCI (HR = 3.51, $p = 0.013$). They also found that the AA genotype of *rs6265* was associated with methylation at this same CpG, which interacted with the risk of MCI compared to controls, (OR: 1.23, 95%CI: 1.12–1.30, $p = 0.019$), as well as progression from MCI to AD (OR:1.40, 95%CI: 1.20–1.48, $p = 0.003$). These findings are important, as distinguishing between

individuals that have stable MCI and those that progress to dementia, brings further understanding of the relationship between DNA methylation and dementia progression.

Within the *BDNF* gene region there are thought to be 11 functional exons, which include 9 different promoter regions [66]. In our study we have only looked at one area of promoter I. Other promoter regions could be of interest, for example, promoter IV, where differences in methylation have been found to be associated with other neurological disorders [67, 68]. Another option beyond *BDNF* methylation could be in epigenome wide association studies (EWAS), where instead of looking at one defined genomic region of ~20 CpG units, they offer data on hundreds of thousands of sites across the genome. This is an important consideration as it allows the entire epigenome, hence the epigenetic dysregulation of potentially multiple genes, to be analysed at once.

As mentioned, previous studies were case-control in design, and included individuals already diagnosed with dementia. Case-control studies are instrumental in bringing understanding to the relationship between blood-based DNA methylation and the disease state, and perhaps useful in diagnosing dementia. However, whether these and other DNA methylation differences can also be detected prior to the appearance of dementia symptoms, remains to be determined. Thus longitudinal studies like ESPRIT with stored biospecimens, that include ongoing cognitive examination, will prove very useful for future studies in this field.

Another future consideration, particularly for studies that examine CpG sites which share probes with EWAS arrays, is to look at the correlation between blood-based DNA methylation and brain DNA methylation. This will bring a deeper understanding to the relationship between blood based DNA methylation biomarkers of disease, and DNA methylation related neuropathophysiology in the brain [13]. This can be done using a publicly available online tool which compares DNA methylation at specific sites, between blood and brain tissues. The tool was created using 450K Illumina methylation data from both blood and multiple brain regions,

and included participants who were neuropathologically unaffected, as well those that had a range of neuropathology [69]. In our study, CpG_26 is the same genomic site as a probe on the 450K Illumina Array (cg15462887). Using the tool, it can be seen that blood-based methylation at this site is positively correlated to methylation of the superior temporal gyrus in the brain ($r = 0.29$, $p = 0.01$) [69]. A high correlation between blood and brain tissue methylation at CpGs associated with dementia, could suggest that blood is an ideal surrogate tissue for investigating dementia related methylation changes in the brain at those specific CpG sites.

CONCLUSION

The results of this study provide little support for methylation of the exon I promoter region of *BDNF* in blood and buccal as a preclinical or diagnostic biomarker of dementia. Further, equivalence testing of null observations indicated that these findings were statistically equivalent to zero, further supporting the overall conclusion of no association. Recommendations for further studies in this area include large longitudinal cohort studies which investigate DNA methylation patterns across the entire epigenome. Such studies should collect biological samples across multiple time points to investigate dynamic methylation in regard to disease progression. Ongoing cognitive assessment will enable clear distinctions to be made between cognitively healthy participants, individuals with MCI, and those with dementia. Importantly, covariate factors, including genetic variation, need to be considered and included in statistical analysis. DNA methylation in its very nature is known to be influenced by not only environmental, but also genetic factors. If an epigenetic biomarker can be identified with sufficient sensitivity and specificity, it could be used for more accurate and less invasive dementia diagnosis. Such a biomarker could also permit preclinical dementia screening and may be useful when monitoring the effectiveness of intervention treatments in individuals with early clinical symptoms, or for those participating in studies of new therapeutic agents.

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Table 1. Baseline characteristics of participants according to prevalent and 14 year incident dementia (prevalent analysis: n=769, incident analysis n=749)

Baseline characteristic	Prevalent Dementia		P	Incident dementia		P
	No	Yes		No	Yes	
	n = 749	n = 20		n = 679	n = 70	
	(97.4%)	(2.6%)		(90.7%)	(9.3%)	
	Mean (SD)			Mean (SD)		
Age	72.95 (5.40)	80.4 (6.75)	<0.0001	72.69 (5.35)	75.43, (5.25)	<0.0001
	n (%)			n (%)		
Female	422 (56.3%)	11 (55%)	0.90	383 (56.4%)	39 (55.7%)	0.91
Male	327 (43.7%)	9 (45%)		296 (43.6%)	31 (44.3%)	
At least 1 *APOE*-ε4 allele	157 (20.9%)	9 (45%)	0.01	130 (19.1%)	27 (38.5%)	<0.0001
Lower education	512 (68.4%)	12 (60%)	0.43	465 (68.5%)	47 (67.1%)	0.82
Low MMSE, ≤ 26 [1]	235 (31.5%)	20 (100%)	<0.0001	204 (30.1%)	31 (44.9%)	0.01
Ever smoker [2]	311 (42.1%)	5 (27.2%)	0.22	278 (41.5%)	33 (48.5%)	0.26
Heavy alcohol use, ≥ 24 g/day [3]	158 (21.6%)	1 (5%)	0.07	141 (21.2%)	17 (25.4%)	0.43
Obese, BMI ≥ 30 [4]	67 (9%)	3 (15%)	0.35	62 (9.2%)	5 (7.1%)	0.55
Depression, CESD16+ [5]	222 (30%)	4 (21.1%)	0.40	192 (28.6%)	30 (42.9%)	0.01
Diabetes[6]	74 (9.9%)	4 (20%)	0.14	61 (8.9%)	13 (18.8%)	0.009

Data not available for [1]3, [2]13, [3]17, [4]1, [5]8, and [6]1 participants.

APOE, Apolipoprotein E; BMI, body mass index; CESD16+, Center for Epidemiologic Studies Depression scale with a score of 16 or over; MMSE, mini mental state examination

Table 2. Multivariate adjusted[1] logistic regression of model for select units of blood-based methylation

	Prevalent dementia				Incident dementia				All dementia cases[2]			
CpG Unit	OR	SE	P	Adjusted P	OR	SE	P	Adjusted P	OR	SE	P	Adjusted P
6	0.62	0.15	0.04	0.22	0.75	0.10	0.04	0.42	0.72	0.09	0.008	0.09
7 8 9	0.60	0.15	0.04	0.22	0.86	0.12	0.30	0.83	0.81	0.10	0.09	0.47

[1]Adjusted for sex, age, education, and *APOE-ε4* genotype.

[2]Diagnosis of dementia at baseline or at any time over follow-up.

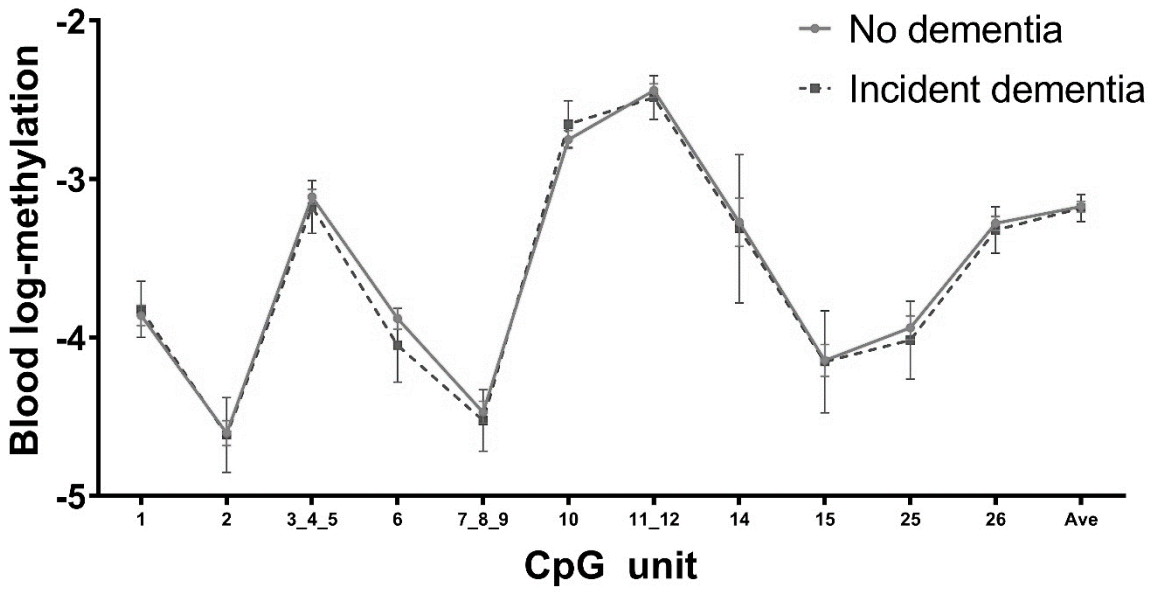

Figure 1. Unadjusted blood log-methylation levels of incident dementia (n=70) vs control participants (n=679) in *BDNF* exon I promoter region. Ave indicates average methylation where all methylation data was present across all CpGs (n=522). Error bars indicate 95% confidence intervals.

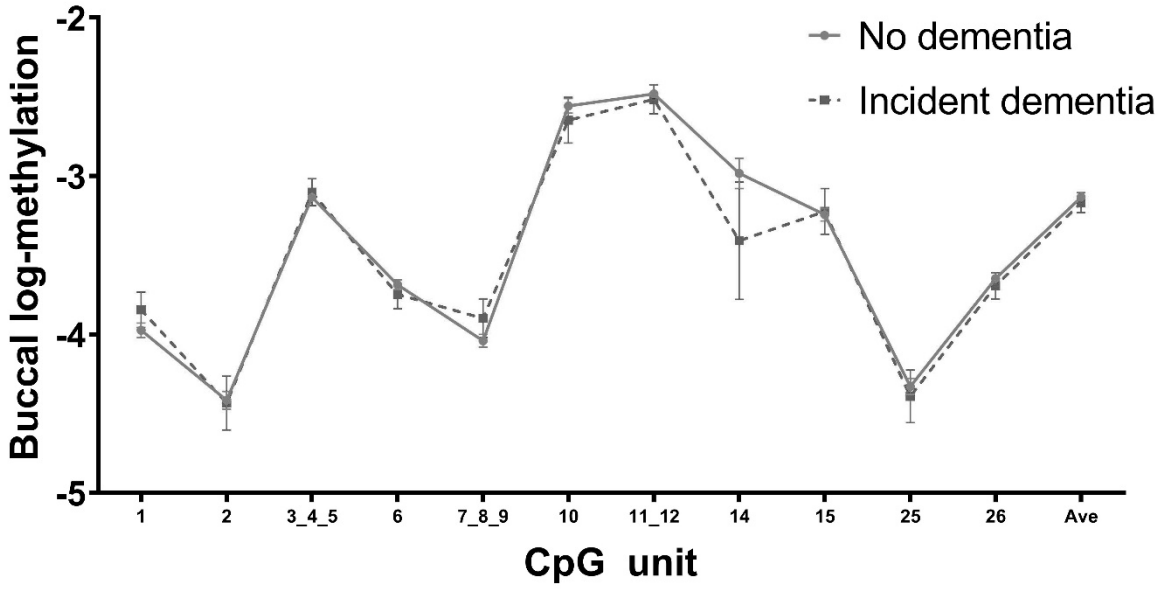

Figure 2. Unadjusted buccal log-methylation levels of incident dementia (n=97) vs control participants (n=965) in *BDNF* exon I promoter region. Ave indicates average methylation where all methylation data was present across all CpGs (n=835). Error bars indicate 95% confidence intervals.

SUPPLEMENTART MATERIAL

Is peripheral *BDNF* promoter methylation a preclinical biomarker of dementia?

Peter D. Fransquet, Karen Ritchie, Vania Januar, Richard Saffery, Marie-Laure Ancelin, Joanne Ryan

Table S1. Genomic Location of CpG's analysed for DNA methylation

CpG Unit	Genomic location (hg19)
CpG 1	CpG11:27,744,252
CpG 2	CpG11:27,744,245
CpG 3.4.5	CpG11:27,744,224 CpG11:27,744,221 CpG11:27,744,219
CpG 6	CpG11:27,744,210
CpG 7.8.9	CpG11:27,744,202 CpG11:27,744,199 CpG11:27,744,196
CpG 10	CpG11:27,744,184
CpG 11.12	CpG11:27,744,169 CpG11:27,744,163
CpG 14	CpG11:27,744,139
CpG 15	CpG11:27,744,134
CpG 25	CpG11:27,744,054
CpG 26	CpG11:27,744,049

Table S2. *BDNF* genotype frequencies in participants

SNP	n	N (%)		
rs6265	739	GG = 464 (62.8)	AG = 253 (34.2)	AA = 22 (3.0)
rs908867	738	GG = 611 (82.8)	AG = 123 (16.7)	AA = 4 (0.5)
rs1491850	737	TT = 255 (34.6)	CT = 370 (50.2)	CC = 112 (15.2)
rs7103411	401	TT = 235 (58.6)	TC = 152 (37.9)	CC = 14 (3.5)
rs962369	383	AA = 202 (52.7)	GA = 151 (39.4)	GG = 30 (7.8)
rs11030101	399	AA = 107 (26.8)	TA = 220 (55.1)	TT = 72 (18.1)
rs28722151	393	CC = 113 (28.7)	GC = 220 (56)	GG = 60 (15.3)

Table S3. The association between *BDNF* genetic variation and dementia prevalence and incidence

Genotype	Prevalent dementia		P	Adjusted P	Incident dementia		P	Adjusted P
	No	Yes			No	Yes		
	%				%			
rs6265, AG/AA genotype	36	50	0.23	0.74	36.7	38.2	0.80	0.97
rs908867, AG/AA genotype	17.1	20	0.74	0.74	17.3	16.2	0.83	0.97
rs1491850, CT/CC genotype	65.3	70	0.66	0.74	64.7	70.6	0.33	0.97
rs7103411, TC/CC genotype	44.7	20	0.33	0.74	41.5	43.2	0.84	0.97
rs962369, GA/GG genotype	46.9	75	0.26	0.74	46.9	47.2	0.97	0.97
rs11030101, TA/TT genotype	73.1	80	0.73	0.74	73.7	67.6	0.43	0.97
rs28722151, GC/GG genotype	71.1	80	0.66	0.74	71.5	67.6	0.62	0.97

Table S4. Characteristics of participants with buccal samples (n=1062)

Characteristic	Incident dementia		P
	No (90.9%)	Yes (9.1%)	
	Mean (SD)		
Age (mean ,SD)	71.50 (4.43)	73.23 (4.88)	0.0003
	n (%)		
Female	583 (60.5%)	58 (59.8%)	0.89
At least 1 *APOE-ε4* allele[1]	161 (16.9%)	32 (33.3%)	<0.0001
Lower education[2]	597 (62.0%)	58 (59.8%)	0.67
Low MMSE, ≤ 26 [3]	222 (23.1%)	32 (33.3%)	0.03
Ever smoker [4]	372 (39.1%)	38 (39.6%)	0.92
Heavy alcohol use, ≥ 24 g/day [5]	176 (18.7%)	18 (19%)	0.95
Obese, BMI ≥ 30 [6]	71 (7.3%)	13 (13.4%)	0.03
Depression, CESD16+[7]	235 (24.6%)	26 (26.8%)	0.629
Diabetes[8]	59 (6.6%)	14 (14.6%)	0.002

Data not available for [1]12, [2]2, [3]5, [4]14, [5]24, [6]5, [7]9, and [8]7 participants.

APOE, Apolipoprotein E; BMI, body mass index; CESD16+, Center for Epidemiologic Studies Depression scale with a score of 16 or over; MMSE, mini mental state examination

Table S5. Multivariate adjusted logistic regression of models for select units of buccal-based methylation and dementia incidence.

		Model 1[1]				Model 2[2]			
CpG Unit	***N***	**OR**	**SE**	**P**	Adjusted P	**OR**	**SE**	**P**	Adjusted P
1	1049	1.36	0.24	0.09	0.32	1.34	0.24	0.11	0.34
7_8_9	1049	1.50	0.28	0.03	0.16	1.57	0.30	0.02	0.14
14	848	0.84	0.06	0.02	0.16	0.85	0.06	0.03	0.14

[1]Adjusted for sex, age, education, and *APOE-ε4* genotype.

Table S6. Correlation between blood and buccal *BDNF* DNA log-methylation for individuals with both samples (n=383)

CpG Unit	**n**	**Correlation coefficient, r**	**p**	Adjusted P
1	379	-0.05	0.35	0.55
2	380	0.06	0.23	0.42
3_4_5	354	0.03	0.61	0.75
6	378	-0.11	0.04	0.22
7_8_9	383	-0.06	0.23	0.42
10	348	-0.08	0.13	0.42
11_12	365	0.03	0.57	0.75
14	227	0.09	0.16	0.42
15	377	-0.11	0.04	0.22
25	382	-0.01	0.79	0.87
26	380	-0.01	0.87	0.87

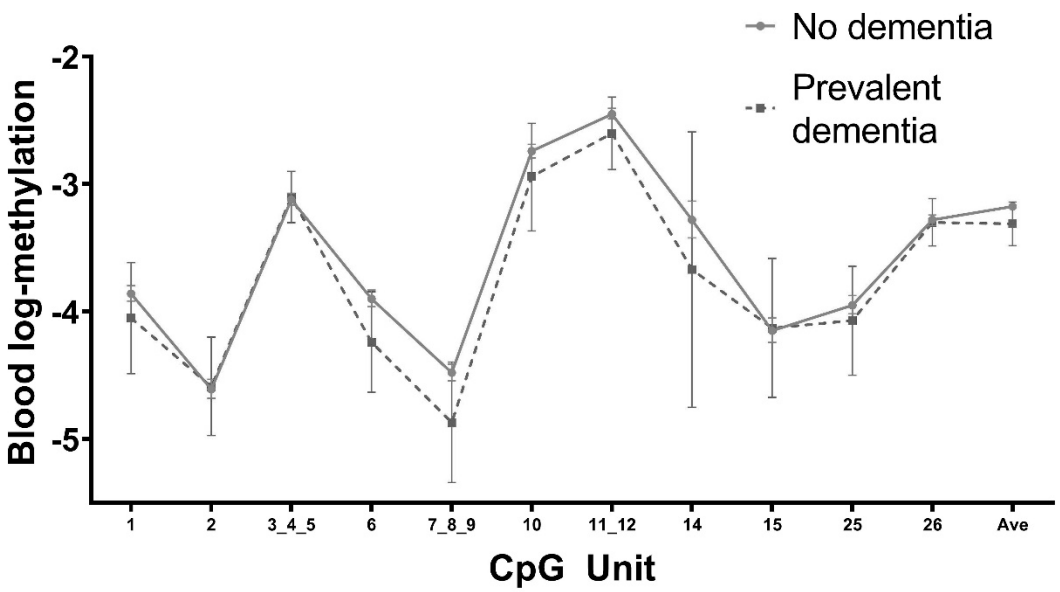

Figure S1. Unadjusted blood log-methylation levels of prevalent dementia (n=20) vs control participants (n=749) in *BDNF* exon I promoter region. Ave indicates average methylation where all methylation data was present across all CpGs (n=522). Error bars indicate 95% confidence intervals.

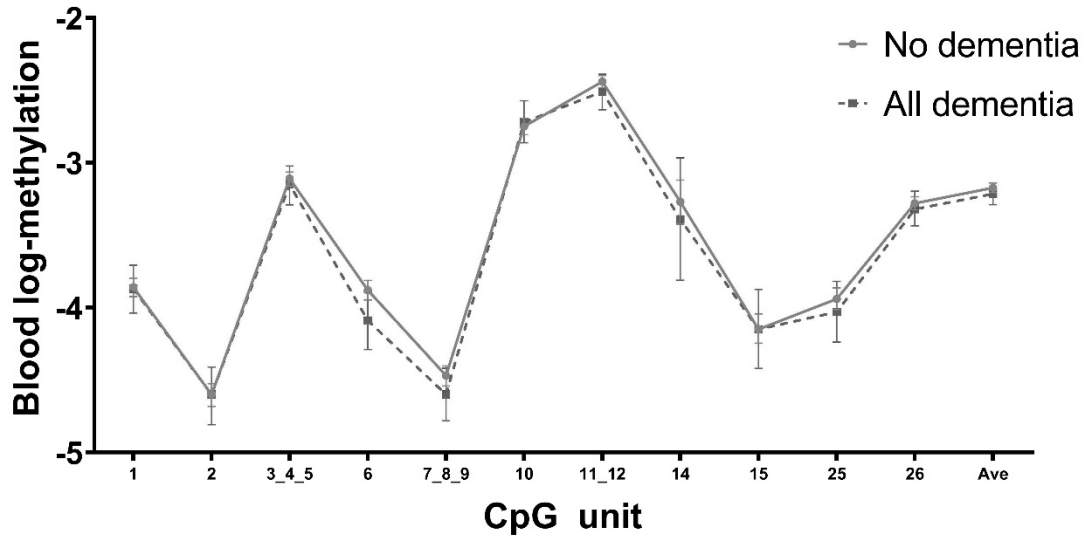

Figure S2. Unadjusted blood log-methylation levels of all cases of dementia (prevalent + incident) (n=90) vs control participants (n=679) in *BDNF* exon I promoter region. Ave indicates average methylation where all methylation data was present across all CpGs (n=522). Error bars indicate 95% confidence intervals.

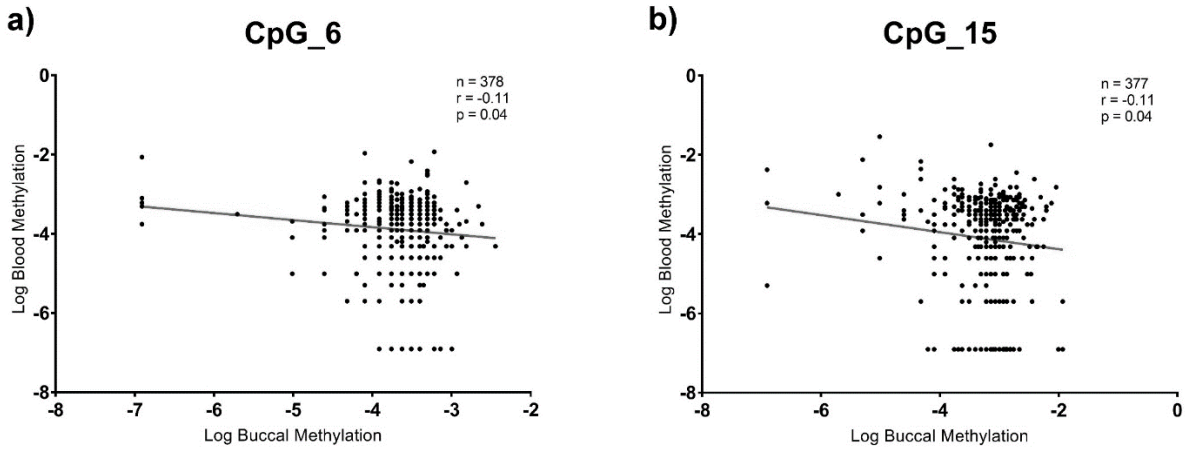

Figure S3. Correlation between interindividual blood log-methylation and buccal log-methylation levels for a) CpG_6 and b) CpG_15.