Received: 30 April 2020 | Revised: 27 May 2020 | Accepted: 27 May 2020 | Published online: 8 July 2020

DOI: 10.1002/dad2.12056

Alzheimer's & Dementia
Diagnosis, Assessment & Disease Monitoring

BLOOD-BASED BIOMARKERS

Blood DNA methylation signatures to detect dementia prior to overt clinical symptoms

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Funding information
National Institutes of Health, Grant/Award Number: U01AG029824; National Health and Medical Research Council, Grant/Award Numbers: 334047, 1127060; Victorian Cancer Agency; NHMRC, Grant/Award Number: APP1135727

Abstract

Introduction: This study determined whether blood DNA methylation (DNAm) patterns differentiate individuals with presymptomatic dementia compared to controls.

Methods: DNAm was measured in 73 individuals prior to dementia diagnosis and 87 cognitively healthy controls matched for age, sex, smoking, education, and baseline cognition. DNAm was also measured at 3 years follow-up in 25 dementia cases, and 24 controls.

Results: Cases and controls differed in DNAm (unadjusted $P < .01$) at the time of diagnosis (n = 28,787 probes), and pre-diagnosis (n = 15,111 probes), with cg01404610 (*General transcription factor IIA subunit 1* gene) significant after correction for multiple testing. Overall, 1150 probes overlapped between analyses (methylation differences from −10.6% to +11.0%), and effect sizes increased from pre-diagnosis to diagnosis.

Discussion: Discernible blood DNAm signatures are in dementia cases before the appearance of overt clinical symptoms. Blood-based methylation may serve as a potential biomarker of dementia, but further investigation is needed to determine their true clinical utility.

KEYWORDS
biomarker, dementia, epigenome-wide association study, methylation, pre-diagnosis

1 | INTRODUCTION

Dementia is a neurodegenerative disease with no cure, preventive intervention, or treatment to slow onset. It involves a loss of cognitive abilities, primarily memory loss, that impacts an individual's independence in day-to-day functioning.[1] An early dementia diagnosis is difficult to obtain due to the often gradual onset in symptoms that are nonspecific and complex.[2] As a result, a dementia diagnosis is often made in the late stages of the disease, after a multitude of visits to general practitioners and specialists for comprehensive cognitive

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screening, blood tests, analysis of cerebrospinal fluid constituents, and brain imaging, and by ruling out other underlying conditions which can present with overlapping symptoms.[3] Further, a definitive diagnosis of the most common cause of dementia, Alzheimer's disease (AD), can only be determined from an autopsy.[4]

Objective biomarkers for dementia are severely lacking.[5] Identification of definitive diagnostic biomarkers would permit an earlier diagnosis, which would allow for individuals diagnosed to make decisions about their future (eg, medical, legal, and financial) while they still have the cognitive capacity.[6] Accurate biomarkers of dementia in the preclinical, asymptomatic stage may be crucial to the development of effective treatment and prevention strategies.[7] They would facilitate effective recruitment into clinical trials at an earlier time-point of the disease, which could then lead to early disease interventions.

There is increasing interest in the potential of epigenetic biomarkers in blood to facilitate early diagnosis of disease.[8] Epigenetics refers to molecular modification of DNA that can regulate gene expression without altering the DNA sequence.[9] Epigenetic mechanisms have been implicated in the etiology of dementia.[10-12] Recent evidence suggests that DNA methylation (DNAm) signatures may also be detected in blood that differentiates individuals with dementia compared to those without.[13] However, at present the degree to which blood-based DNAm patterns have prognostic utility in dementia, prior to the appearance of clinical symptoms, remains unclear.

This study investigated whether DNAm differences could be identified in the blood of individuals several years prior to a dementia diagnosis (cases), compared to individuals who remain cognitively healthy (controls). Second, this study investigated whether these same DNAm patterns differentiated individuals with dementia at the time of diagnosis and without.

HIGHLIGHTS

- Blood-based DNA methylation differs between dementia cases and controls.
- A unique methylation signature is also present in presymptomatic dementia.
- Effect sizes were greater at diagnosis than presymptomatic.
- Gene regions identified have been previously implicated in neurological functioning.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors performed a systematic literature review using MEDLINE and EMBASE databases, following PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) guidelines. Studies needed to have measured DNA methylation in blood in individuals with dementia or prior to diagnosis, and to compare this to a cognitively healthy control group.
2. **Interpretation**: Building on existing evidence, our findings show that differences in blood DNA methylation patterns are found between individuals with dementia and controls. However, novel findings from our work indicate that the DNA methylation signature in dementia cases can be detected at least 3 years prior to the appearance of clinical dementia symptoms.
3. **Future directions**: This study indicates the potential for a blood DNA methylation biomarker of dementia in the presymptomatic stage. Independent replication and validation of these findings in a well-defined cohort consisting of dementia cases, presymptomatic dementia, and cognitively healthy controls is now required.

2 | METHODS

2.1 | Study sample

Data and biospecimens for this study came from the Aspirin in Reducing Events in the Elderly (ASPREE) cohort, which has been detailed previously.[14] In brief, ASPREE is a randomized, double-blind, placebo-controlled study of the effects of low-dose aspirin on disability-free survival in 19,114 older community-dwelling individuals. Participants were recruited from general practice (in Australia, n = 16,703) or clinical trial networks (in the United States, n = 2411). Exclusion criteria included a previous dementia diagnosis, or a score of < 78 on the Modified Mini-Mental State Examination (3MS), which excluded severe cognitive impairments. The study was in accordance with the Declaration of Helsinki 2008 revision, the National Health and Medical Research Council (NHMRC) Guidelines on Human Experimentation, the federal patient privacy (Health Insurance Portability and Accountability Act [HIPAA]) law, the International Conference for Harmonisation Guidelines for Good Clinical Practice, and the Code of Federal Regulations. The trial ran for a median of 4.7 years. Neurocognitive assessment was conducted in participants at baseline, 1-year, and 3-year follow-up time points. These included the 3MS,[15] a modified version of the Mini-Mental State Examination which assesses global cognitive function,[16] with a score of less than 78 out of 100 indicative of cognitive impairments; the Symbol Digit Modalities Test (SDMT),[17] a timed test of attention and processing speed; the Controlled Oral Word Association Test (COWAT)[18] using the letter F, which assess language/verbal fluency by asking participants to name as many words starting the letter "F" in a given time frame; and the Hopkins Verbal Learning Test Revised (HVLT-R)[19] to measure delayed recall memory on a scale of 0 to 12, with higher scores indicating better test performance.[20]

2.2 | Dementia diagnosis in Aspirin in Reducing Events in the Elderly (ASPREE)

Over the trial, individuals with a score of < 78 on the 3MS, a drop of > 10.15 on the 3MS, who commenced dementia medication, or who had a dementia diagnosis, were considered possible dementia cases. These individuals underwent additional cognitive assessments and physical function questionnaires. An international panel of clinical specialists, including neurologists, neuropsychologists, and geriatricians from Australia and the United States reviewed the cognitive and functional assessments, medical records, and clinical diagnosis information (as well as the results of blood tests and brain scans when available) to reach a diagnosis consensus based on Diagnostic and Statistical Manual for Mental Disorders, American Psychiatric Association (DSM-IV) criteria.[21] From the 19,114 participants, 964 triggered further assessments for dementia, and 575 individuals were adjudicated as having dementia by the end of the trial period.

2.3 | Selected subsample

This substudy consisted of 160 Australian-based participants. Dementia cases and cognitively healthy controls were selected from individuals who provided blood samples at study randomization (baseline) when all individuals were cognitively healthy, and based on their dementia status at the year 3 follow-up. Controls were required to be without a dementia diagnosis over the duration of the trial, while dementia cases needed to have triggered further dementia assessment (which resulted in subsequent adjudication as dementia) at least 1 year after baseline and within 9 months of providing their 3-year blood sample. The cases (n = 73) and controls (n = 87) were additionally matched on age, self-reported sex, education, smoking status, and cognitive function at baseline. Forty-nine participants who provided samples at inclusion had also provided samples at follow-up (25 cases and 24 controls), and were included in the follow-up analysis (diagnosed dementia vs controls).

2.4 | Quality control and methylation profiling

DNA from peripheral blood was extracted using Qiagen DNeasy Blood & Tissue Kits. Cytosine-phosphate-guanine (CpG) probe methylation was measured using the Illumina Infinium MethylationEPIC BeadChip (EPIC) at the Australian Genome Research Facility, Melbourne Victoria (http://www.agrf.org.au/).

All data were analyzed using R version 3.5.1 following an adapted Bioconductor workflow protocol.[22] Quality control found no failed genomic position probes, defined as both the methylated and unmethylated channels reporting background signal levels (ie, no probe detected), to a significance level of $P < .002$ ("detection," R package Minfi).[23] Sample data were normalized using subset quantile normalization ("preprocessQuantile").[24] Self-reported sex was confirmed as concordant with biological sex using "getSex" of the minfi R package. Probes that failed in at least one sample, with known single nucleotide polymorphisms, sex chromosome probes, and cross-reactive probes,[25] were removed from the data set (Table S1 in supporting information). A total of 626,793 probes were available for further analysis. All probes are mapped to the human genome assembly GRCh37 (hg19). Data were transformed into "M values" (log2 of array intensities at each probe) and "Beta values" (average DNAm within a sample as a measure between zero (probe is unmethylated) and one (100% methylated).

2.5 | Statistical analysis

Analysis was undertaken for both pre-diagnosis "cases" versus "controls" (baseline blood samples) and dementia cases versus controls (follow-up blood samples).

A principal component analysis was used to determine the degree to which technical or environmental variables influenced variability of methylation in the data set ("WGCNA";[26] Figures S1–S4 in supporting information). Differentially methylated probes were obtained using R package "limma" linear regression models, adjusting for possible confounding variables.[27] Models included dementia status, age, sex, and methylation measurement batch in both analyses. Observations were adjusted for false discovery rate by the Benjamini and Hochberg (BH) method.

Stata 15 was used for probe wise *t*-tests between cases and controls, using methylation beta values from top probes in aforementioned models (ie, highest significance, or greatest change in methylation in cases compared to controls). Regression analysis was carried out to determine whether differences seen remained after adjusting for common confounding factors, being age, sex, and methylation measurement batch. Probe nomenclature is labelled as the Illumina EPIC probe name, followed by the methylated dinucleotide, chromosome, and genomic location (bp), for example, "cg01404610, CpG14:81687455."[28]

As the aim of this study's analyses was discovery, further analysis took into account all probes which reached non-BH adjusted *P*-value of ≤.01, chosen as a conservative cut-off compared to $P < .05$. Differentially methylated region (DMR) analysis was carried out using "DMRcate" to identify gene regions that contained multiple significant probes.[29] DMRcate ranks DMRs by Stouffer's test statistic, which is used to combine the results from several independent tests bearing upon the same overall hypothesis.[30] Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used in gene pathway analysis, carried out using the "gometh" function from the missMethyl R package.[31]

3 | RESULTS

3.1 | Baseline participant characteristics

Baseline characteristics of the 160 participants, 73 pre-diagnosis cases, and 87 controls are shown in Table 1. Only average COWAT scores differed between the two groups.

TABLE 1 Baseline characteristics of participants (n = 160)

Participant characteristics	Controls (n = 87)	Pre-diagnosed Dementia (n = 73)	*P*
Age, mean, (SD)	76.4 (4.6)	77.6 (5.1)	0.11
Sex, n (% ♀)	50 (57.5)	42 (57.5)	0.99
Years to incident dementia mean (SD)	—	3.7 (1.1)	NA
	n (%)		
Smoking			
Current	2 (2.3)	0 (0)	0.42
Past	36 (41.4)	32 (43.8)	
Never	49 (56.3)	41 (56.2)	
Education			
< = 12 years	60 (69)	43 (58.9)	0.19
> 12 years	27 (31)	30 (41.1)	
	mean, (SD)		
3MS	93.2 (4.8)	92.1 (4.4)	0.14
SDMT	36.2 (9.1)	35.0 (8.4)	0.39
COWAT	12.8 (3.6)	14.1 (4.0)	0.03
HVLT-R delayed recall[a]	8.2 (1.9)	7.8 (1.9)	0.2

Abbreviations: 3MS, Modified Mini-Mental State Examination; COWAT, Controlled Oral Word Association Test; HVLT-R, Hopkins verbal learning Test Revised; SD, standard deviation; SDMT, Symbol Digit Modalities Test.
[a]Data not available for one participant.

The median time between baseline and the follow-up visits was 2.98 years. At follow-up, 49 of the aforementioned participants, 24 controls, and 25 cases with incident dementia also provided blood samples for DNAm analysis (Figure S5 and Table S2 in supporting information). Education level differed between dementia cases and controls ($P = .005$). Cases also had significantly lower scores on the 3MS, SDMT, and HVLT-R delayed recall.

3.2 | Differentially methylated probes between dementia cases prior to diagnosis and controls

A model adjusting for age, sex, and methylation batch showed one probe that passed BH adjustment for multiple testing (cg01404610, CpG14:81687455, Δ = +0.87%, BH Adj.*P* = .018, Figure 1a). This probe is within a CpG island (a genomic region rich in CpGs) of an intronic promotor region of general transcription factor IIA subunit 1 (*GTF2A1*).

A total of 15,111 methylation probes (2.4% of total probes) differed between dementia participants prior to diagnosis and controls at $P \leq .01$ (adjusting for age, sex, and methylation batch only). Of these the majority 8729 (57.8%) had higher methylation in pre-diagnosed cases compared to controls. The top 10 probes ranked by *P*-value can be seen in Table 2. The largest methylation difference between pre-diagnosis dementia cases and controls was 7.89% lower methylation at cg03635442, CpG8:49427283 (SE:2.97%, $P = .009$, Adj.$P = .009$, Figure 1b), within a CpG island upstream of *LOC101929268*, an uncharacterized RNA gene. Other top probes ranked by higher and lower effect sizes ($P < .05$) can be seen in Table S3 and Table S4 in supporting information, respectively.

3.3 | Differentially methylated probes between dementia cases at diagnosis and controls

When comparing dementia cases versus controls, no probes passed BH adjustment for multiple testing. When adjusting for age, sex, and methylation batch only, a total of 28,787 probes (4.6% of total probes) were found to be differentially methylated between controls and those with a dementia diagnosis ($P < .01$), with the majority (14,879, 57.7%) being lower in methylation compared to controls. Top probes ranked by *P*-value, adjusted for age, sex, and batch, can be seen in Table 3, and top probes ranked by effect size ($P < .05$) can be seen in Table S5 in supporting information (higher methylation) and six (lower methylation).

The largest methylation difference was a lower methylation at cg08642068, CpG20:31591776, Δ = -15.27%, SE: 4.37%, $P = 0.001$, Adj.$P = 0.001$ (Figure 1f, Table S6 in supporting information). This probe is within the Sad1 and UNC84 domain containing 5 (*SUN5*) gene.

3.4 | Common differentially methylated probes between pre-diagnosis and diagnosed dementia analyses

There were 1150 differentially methylated probes (DMPs) common between the two analyses, when adjusting for age, sex, and batch ($P < .01$). The top 10 common probes, ranked by significance level in both analyses, can be seen in Table S7 in supporting information. In all cases, the direction of association was the same, with a generally larger effect size seen when comparing cases and controls at diagnosis, compared to pre-diagnosed cases and controls. Of these, the largest methylation difference was a lower methylation in dementia diagnosis compared to controls, at cg06354780, CpG1:77296123, (−9.13%, SE:1.13%, $P < .0001$); prior to dementia diagnosis this difference was less than half (−3.41%, SE:1.21%, $P = .005$). This probe is situated between the long intergenic non-protein coding RNA 2567 (*LINC02567*), and ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 (*ST6GALNAC5*) genes.

3.5 | Differentially methylated regions

Regions of the genome which were found to be differentially methylated between cases and controls, differentially methylated regions (DMRs), were defined if they included at least three probes that were significantly different ($P < .01$) within the above DMP analyses, and had an average effect size of at least 0.5% methylation difference across the region. At baseline, there were 377 DMRs associated with pre-diagnosed dementia, which contained at least one probe with a minimum false discovery rate (FDR) of $P < .01$ (File S1 in supporting

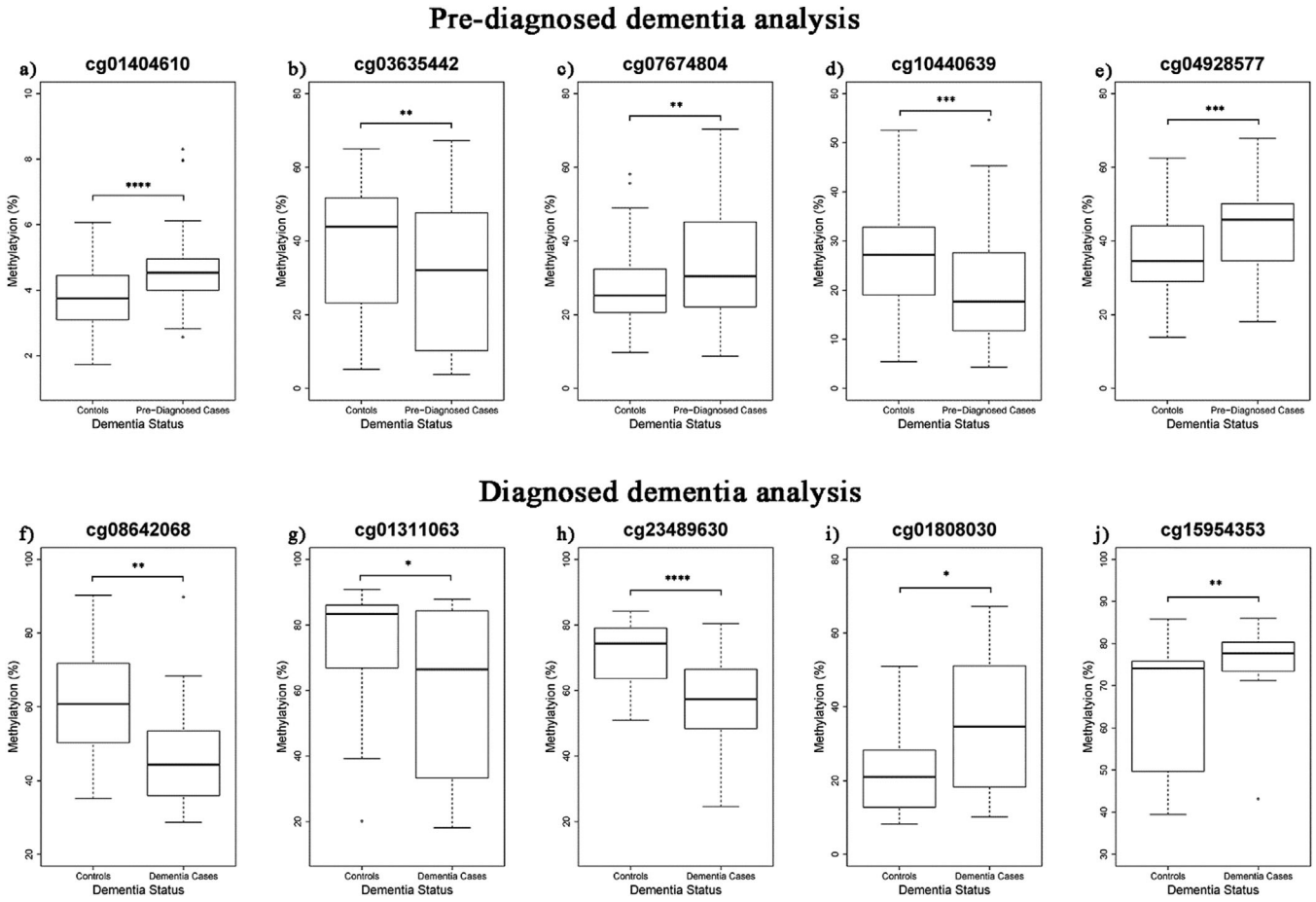

FIGURE 1 Top differential probes comparing methylation of pre-diagnosed dementia (n = 73) versus controls (n = 87; a–e), and diagnosed dementia (n = 25) versus controls (n = 25; f–j). a, Probe CpG14:81687455, cg01404610 was the only probe to pass Benjamini and Hochberg adjustment for multiple testing (Δ −0.87%, SE:0.15, $P < .0001$). b, CpG8:49427283, cg03635442, Δ −7.89%, SE:2.97, $P = .009$). c, CpG13:108739184, cg07674804, Δ +6.46%, SE:1.75, P = .002). d, CpG17:154499, cg10440639, Δ −6.40%, SE:1.73, $P = .0003$). e, CpG12:63696410, cg 04928577, Δ +6.11%, SE:1.75, $P = .0006$). f, CpG20:31591776, cg08642068, Δ −15.27%, SE:4.37, $P = .001$). g, CpG2:131058184, cg01311063, Δ −14.96%, SE:6.33, $P = .02$). h, CpG19:44645078, cg23489630, Δ −14.61%, SE:3.49, $P = .0001$). i, CpG22:45809952, cg01808030, Δ +11.71%, SE:4.61, $P = .02$). j, CpG17:5403337, cg15954353, Δ +11.36%, SE:3.48, $P = .002$). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P \leq .0001$

information). These DMRs included between 3 and 21 methylation probes, and covered regions between 7 and 2461 base pairs (bp) long. However, none of these DMRs passed adjustment for multiple testing. The DMR with the largest average methylation difference was at vault RNA 2-1 (*VTRNA2-1*), from 7 probes within 384 bp region (Δ = -4.53%, Stouffer Adj.$P = .52$).

At follow-up, there were 1906 DMRs associated with dementia which contained at least one probe with a minimum FDR of $P < .01$. These DMRs included between 3 and 56 probes, and regions of 12 to 3783 bp in length (File S1). Eighty-four DMRs passed Stouffer adjustment ($P < .05$). The most significant DMR overlapped the integrin subunit alpha E (*ITGAE*) and nuclear cap binding dubunit 3 (*NCBP3*) gene regions (13 probes in a 2894 bp region, chr17:3,704,471-3,707,364, Δ = −4.48%, Stouffer Adj.$P = .005$). The largest average methylation difference was seen in a CpG island preceding the kinesin family member 25 antisense RNA 1 (*KIF25-AS1*) gene (5 probes in a 269 bp region, chr6:168,393,930-168,394,198, Δ = -6.32%, Stouffer Adj. P = 0.038).

Comparisons of DMRs between analyses were made using the DMR's exact genomic coordinates, resulting in 21 DMRs common between pre-diagnosed and diagnosed dementia (Table 4). All common DMRs had the same direction of association between baseline and follow-up, with all but two being lower in the same direction over time (*HOXA7* average methylation difference −1.05% at baseline but −0.92% at follow-up, and *ADAM12* average methylation difference −2.16% at baseline but −1.90% at follow-up).

3.6 | Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

Pathways associated with pre-diagnosed dementia and dementia cases can be seen in Table 5. Pre-diagnosed dementia was only associated with one pathway from each database when adjusting for FDR, GO:0005654, nucleoplasm (1438/3123 methylated genes in

TABLE 2 Top 10 differentially methylated probes ranked by significance*, comparing baseline blood sample methylation between pre-diagnosis dementia cases (n = 73) and controls (n = 87)

CpG Unit	Genomic location	Gene context	Pre- diagnosis dementia	μ (%)	Δ	SE	*P*
cg01404610	Chr14:81687455	*GTF2A1*	No	3.74%	+0.87%	0.15%	<.0001
			Yes	4.61%			
cg04298511	Chr7:70159330	*AUTS2*	No	5.26%	−1.33%	0.31%	<.0001
			Yes	3.93%			
cg12679980	Chr5:43557583	*<PAIP1 > NNT-AS1*	No	15.01%	+2.59%	0.48%	<.0001
			Yes	17.61%			
cg16274893	Chr14:69204080	*<RAD51B > ZFP36L1*	No	73.48%	−1.82%	0.36%	<.0001
			Yes	71.66%			
cg22871559	Chr1:206730254	*RASSF5*	No	3.53%	+0.43%	0.09%	<.0001
			Yes	3.96%			
cg19217964	Chr16:63402222	*<CDH8 > CDH11*	No	57.12%	+2.34%	0.48%	<.0001
			Yes	59.46%			
cg13616097	Chr22:46372744	*WNT7B*	No	9.36%	−2.33%	0.53%	<.0001
			Yes	7.03%			
cg02608511	Chr8:71135222	*NCOA2*	No	78.51%	−1.84%	0.37%	<.0001
			Yes	76.67%			
cg14609448	Chr21:34896882	*GART*	No	85.93%	−1.53%	0.31%	<.0001
			Yes	84.40%			
cg09251344	Chr15:67814243	*C15orf61*	No	3.82%	+0.43%	0.09%	<.0001
			Yes	4.25%			

Note: These results remained significant after adjustment for age, sex, and methylation processing batch.
*Ranked by limma analysis significance level, μ, mean methylation %; **Δ**, mean methylation difference.
Gene context " > " represents gene downstream from probe, " < " represents gene upstream from probe, otherwise probe sits within the gene.

pathway, FDR Adj.*P* = .001), and hsa:04360, Axon guidance (114/181 methylated genes in pathway, FDR Adj.*P* = .02). At follow-up, there were three pathways associated with dementia using GO and 15 using KEGG. The top dementia associated KEGG pathway was also hsa:04360 Axon guidance (133/181 methylated genes in pathway, FDR Adj.*P* = .001).

4 | DISCUSSION

Given that approximately 60% of dementia cases go undiagnosed, it is clear that there is an urgent need for definitive biomarkers of the disease.[32] Here we have identified DNAm signatures in the blood of apparently healthy older individuals, which differentiate those individuals who remain cognitively healthy from those who are diagnosed with dementia 3 years later. Some probes, gene regions, and pathways that we identified appear biologically relevant, suggesting that these may not only serve as biomarkers, but may potentially be involved in the pathogenesis of the disease. Further, the consistency in many of the main findings, and the increasing effect size between the analysis of pre-diagnosed and diagnosis DNAm profiles, suggests a potential relationship between these DNAm signatures and the appearance of disease symptoms and thus, progression. Only one probe (cg01404610) passed adjustment for multiple testing in pre-diagnosis analysis. It is within the *GTF2A1* gene, a part of the TFIID-DNA complex which is involved in transcription initiation.[33] This is a novel finding, and the exact role in dementia or biological processes disrupted in dementia, remains unclear.

Despite not reaching significance after correction for multiple testing, there were several other individual probes in gene regions previously implicated in dementia. For example, cg16474696, CpG19:13875014 (Δ +5.99%, pre-diagnosis cases), is within a CpG island upstream from methylthioribose-1-phosphate isomerase 1 (*MRI1*) gene. MRI1 is an enzyme involved in the methionine salvage pathway, which results in the formation of the amino acid methionine via reprocessing cellular metabolites that contain sulphur.[34] A previous study (n = 11) also showed increased methylation in the same *MRI1* region at a nearby probe (only 98 bp away) in the brain (Δ +25.8%) and blood (Δ +28.4%; cg25755428) to be associated with Parkinson's disease.[35] Further details of findings which align with previously identified genes involved in dementia are given in Appendix S1 in supporting information.

Additionally, some of the common DMRs are in the regions of genes which have previously been implicated in key pathological processes of dementia. Amyloid beta (Aβ) build-up is one of the hallmark pathologies of AD, and thought to be present prior to clinical manifestation

TABLE 3 Top 10 differentially methylated probes ranked by significance*, comparing follow-up blood sample methylation between diagnosed dementia cases (n = 25) and controls (n = 24)

CpG Unit	Genomic location	Gene context	Dementia	μ (%)	Δ	SE	*P*
cg17750831	chr3: 123304350	<*HACD2* > *MYLK-AS1*	No	2.92%	+0.65%	0.10%	<.0001
			Yes	3.58%			
cg13153264	chr1: 152680502	<*LCE2A* > *LCE4A*	No	51.73%	−7.12%	1.20%	<.0001
			Yes	58.85%			
cg15197125	chr8: 128859546	*PVT1/MYC*	No	53.62%	−6.28%	1.07%	<.0001
			Yes	47.33%			
cg14350179	chr22: 43411101	*PACSIN2*	No	3.24%	+0.75%	0.14%	<.0001
			Yes	3.99%			
cg06823517	chr5: 35484879	<*PRLR* > *SPEF2*	No	79.93%	−3.55%	0.62%	<.0001
			Yes	76.38%			
cg26390944	chr13: 78433828	*EDNRB-AS1*	No	66.32%	−5.19%	0.95%	<.0001
			Yes	61.14%			
cg05868469	chr15: 93128777	<*LINC00930* > *FAM174B*	No	59.28%	+4.77%	0.85%	<.0001
			Yes	64.04%			
cg22455795	chr19: 41109651	*LTBP4*	No	20.67%	+6.78%	1.45%	<.0001
			Yes	27.46%			
cg10491563	chr3: 158378015	*GFM1*	No	53.43%	−11.35%	2.28%	<.0001
			Yes	42.08%			
cg03300589	chr5: 41510820	<*PLCXD3* > *OXCT1*	No	17.65%	−3.32%	0.69%	<.0001
			Yes	14.32%			

Note: These results remained significant after adjustment for age, sex, and methylation processing batch.
*Ranked by limma analysis significance level, μ, mean methylation %; Δ, mean methylation difference.
Gene context " > " represents gene downstream from probe, " < " represents gene upstream from probe, otherwise probe sits within the gene.

of the disease.[36] We observed a DMR in *GULP1* (GULP PTB domain containing engulfment adaptor 1) in both pre-diagnosed and diagnosed dementia cases. Differences in expression of *GULP1* are thought to modify disease progression in AD, as it is an Aβ precursor protein adapter protein, which has a transactive relationship with low-density lipoprotein receptor related protein 1 (LRP1) in clearing apoptotic cells by engulfment in the brain.[37]

Gene pathway enrichment analysis was carried out to observe whether pre-diagnosed and diagnosed dementia associated methylation formed patterns within specific genetically linked biological processes. Within this study we found two KEGG pathways that may be of interest to dementia-related pathology, KEGG:hsa04360 and KEGG:hsa04724. The KEGG:hsa04360 pathway is involved in axon guidance, primarily involved in the encoding of guidance factors including ephrins, netrins, semaphorins, and slits.[38] In our study, this pathway was observed to be associated with both pre-diagnosed dementia (114 of 181 genes containing differential methylation, FDR Adj.*P* = .02), and dementia diagnosis (133 of 181 genes, FDR Adj.*P* = .001). This suggests that the hsa04360 pathway has aberrant methylation earlier than the clinical symptoms of dementia, which becomes more differentially methylated as the disease progresses. Interestingly, several microRNAs, are reported to interact with this pathway, in association with having cataracts.[39] Not only are cataracts an age-related disease, and the leading cause of blindness, but they are also a known risk factor for cognitive decline and dementia.[40] KEGG:hsa04724, is involved in glutamatergic synapse function, and may also be involved in the pathology of dementia. This pathway was found to be differentially methylated only in the diagnosed dementia group (81 of 114 genes, FDR Adj.*P* = .046). Glutamatergic signaling occurs in synapses throughout the nervous system, and is involved in neuronal excitability and neurotransmission.[41] Because of this it is seen as a potential drug target for neurological disorders including dementias.[41] Another study has also recently observed this pathway to have differential expression in association with AD.[42]

The main strength of this study is the comprehensive, in-depth cognitive testing available on all participants from baseline to follow-up, including adjudicated dementia diagnosis. The cognitive screening at baseline ensured that all participants were cognitively healthy at study entry, when the first blood sample was collected. The longitudinal nature of the study with the collection of blood samples at two timepoints (both prior to and at the time of dementia diagnosis) meant we were able to not only carry out a dementia case control analysis, but also compare methylation profiles of cases and controls prior to appearance of clinical symptoms. This provided a unique opportunity to generate pre-clinical methylation profiles and to identify genes that are possibly involved in the progression of dementia. Most other

TABLE 4 Differentially methylated regions (DMR)* found in common between the analysis of pre- and post-diagnosis blood samples

Gene	Genomic location	DMR length (bp)	Number of probes	Time point	Stouffer	Mean DMR Δ %
GULP1	chr2:189156425-189157566	1142	15	Baseline	0.45	−0.89
				Follow-up	0.04	−1.42
SORCS3	chr10:106400565-106401517	953	6	Baseline	0.54	−1.26
				Follow-up	0.42	−1.88
PIEZO2	chr18:11148510-11149470	961	10	Baseline	0.51	−1.56
				Follow-up	0.80	−1.94
DNAH14	chr1:225117076-225117676	601	7	Baseline	0.64	−1.61
				Follow-up	0.23	−2.65
RIBC2	chr22:45809244-45809952	709	13	Baseline	0.68	1.68
				Follow-up	0.43	2.99
FOXG1	chr14:29235904-29236535	632	13	Baseline	0.62	−1.26
				Follow-up	0.21	−1.81
HOXC5	chr12:54425156-54425634	479	3	Baseline	0.32	−1.87
				Follow-up	0.19	−2.46
EPHA6	chr3:96532859-96533824	966	8	Baseline	0.44	−1.44
				Follow-up	0.04	−2.71
HOXA7	chr7:27195602-27196153	552	7	Baseline	0.53	−1.05
				Follow-up	0.29	−0.92
SLC6A2	chr16:55689851-55690418	568	10	Baseline	0.72	−1.02
				Follow-up	0.61	−1.29
MIR199A2	chr1:172113506-172114419	914	9	Baseline	0.64	−0.97
				Follow-up	0.31	−1.46
SYN3	chr22:33453893-33454632	740	10	Baseline	0.82	−0.66
				Follow-up	0.18	−2.51
SLC4A10	chr2:162283705-162284206	502	6	Baseline	0.60	−0.88
				Follow-up	0.43	−1.36
IRX4	chr5:1886956-1887583	628	14	Baseline	0.87	−0.98
				Follow-up	0.73	−1.70
CNTN1	chr12:41221505-41221855	351	7	Baseline	0.58	1.98
				Follow-up	0.03	4.65
ARRDC4	chr15:98503768-98503878	111	7	Baseline	0.46	−0.69
				Follow-up	0.32	−1.04
NOS1	chr12:117798627-117799083	457	3	Baseline	0.46	−1.10
				Follow-up	0.19	−2.59
LOC101929268	chr8:49468684-49469113	430	8	Baseline	0.54	−0.91
				Follow-up	0.21	−1.41
EDIL3	chr5:83680045-83680326	282	7	Baseline	0.52	−1.38
				Follow-up	0.23	−2.29
MARCH3	chr5:126205009-126205081	73	3	Baseline	0.37	−2.03
				Follow-up	0.16	−2.69
ADAM12	chr10:128076910-128076941	32	3	Baseline	0.38	−2.16
				Follow-up	0.47	−1.90

Abbreviations: Bp, basepairs; DMR, differentially methylated region.

*Common DMR is defined here as 21 regions showing the exact genomic location in both pre- and post-diagnosis blood sample analyses.

TABLE 5 Gene pathway analysis using top methylated probes*

Database	Time point	Pathway	Term	Number of genes in pathway	Number of differentially methylated genes	*P*	FDR
GO	Pre-diagnosis	GO:0005654	Nucleoplasm	3123	1438	6.37e-08	0.001
	Diagnosis	GO:0007165	Signal transduction	827	491	4.16e-08	0.0007
		GO:0003779	Actin binding	236	165	9.98e-07	0.007
		GO:0005887	Integral component of plasma membrane	890	499	3.16e-06	0.02
KEGG	Pre-diagnosis	path:hsa04360	Axon guidance	181	114	6.91e-05	0.02
	Diagnosis	path:hsa04360	Axon guidance	181	133	3.67e-06	0.001
		path:hsa04713	Circadian entrainment	97	73	1.02e-04	0.009
		path:hsa05200	Pathways in cancer	531	321	5.76e-05	0.009
		path:hsa05412	Arrhythmogenic right ventricular cardiomyopathy	77	60	7.83e-05	0.009
		path:hsa04020	Calcium signaling pathway	193	128	1.81e-04	0.01
		path:hsa04660	T cell receptor signaling pathway	104	73	2.07e-04	0.01
		path:hsa04010	MAPK signaling pathway	295	189	2.83e-04	0.01
		path:hsa04015	*RAP1* signaling pathway	210	141	4.06e-04	0.02
		path:hsa04921	Oxytocin signaling pathway	153	106	3.98e-04	0.02
		path:hsa04024	cAMP signaling pathway	216	137	9.35e-04	0.03
		path:hsa04014	Ras signaling pathway	232	147	1.15e-03	0.04
		path:hsa04512	ECM-receptor interaction	88	63	1.62e-03	0.045
		path:hsa04072	Phospholipase D signaling pathway	148	101	2.06e-03	0.046
		path:hsa04611	Platelet activation	124	84	1.87e-03	0.046
		path:hsa04724	Glutamatergic synapse	114	81	1.95e-03	0.046

*Probes ≤0.01 after limma model adjustment for age, sex, and batch effects, which included 15,111 in the pre-diagnosis analysis and 28,787 in the diagnosis analysis.

Abbreviations: cAMP, cyclic adenosine monophosphate; ECM, extracellular matrix; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; Rap1, Ras-proximate-1.

studies only focus on dementia cases versus controls.[12] However, while this is a novel study, with an average of nearly 3 years between "cognitively healthy" baseline and dementia diagnosed at follow-up, it is not enough time to detect the very early appearance of dementia related neuropathophysiology, thought to begin decades in advance of symptoms.[43] Because of this, future studies should aim to include younger participants in their mid-adulthood, and follow them longitudinally with initial and regular follow-up samples collected, which could be used to measure DNA methylation. Given most cases of dementia occur later in life (after 70 years), such studies will need to be very long and with regular tracking of cognitive function and dementia assessments.

In comparison to some other DNAm studies of dementia, our groups of cases and controls are relatively large.[13] However, because of the large number of methylation probes measured, and thus the need to correct for multiple testing, the study is underpowered to detect the anticipated small effect sizes (< 10%). While raw, unadjusted results were also reported here, the potential for type 1 errors must be considered, as some of these are likely to be chance findings.

Another limitation to consider was the inability to distinguish cases according to the underlying cause of dementia. Future studies should involve large groups of participants with defined causes of dementia, so disease-specific epigenetic profiles can be generated. In older individuals AD is a common cause of dementia,[44] but it is increasingly recognized that many individuals over the age of 80 years likely have mixed dementia, with a complex combination and manifestation of a spectrum of brain conditions, as well as AD pathology.[45,46] Furthermore, the focus on a white population means the results can not necessarily be generalized to other ethnic groups.[47] Finally, blood is composed of multiple cell types which have different DNAm signatures. No adjustment was made for cell proportions in the analysis because adjusting for cell type removes important biological signatures that may be closely linked to disease etiology.[48] Differing cell types may be a result

of the disease itself, and an easy-to-obtain biomarker should be present regardless of a cell type estimation.

There still remains a large gap between DNA methylation biomarker studies and translation to the clinical setting. Replication and validation of these results are needed to refine dementia-specific DNA methylation signatures. In doing so, a polyepigenetic risk score, of high sensitivity and specificity, could be created for both dementia in general, as well as specific diseases which cause dementia.

5 | CONCLUSION

We have identified a distinct DNAm signature which differentiates individuals with and without dementia. Methylation of some sites, gene regions, and genetic pathways may have functional implications in the pathophysiology of dementia. Furthermore, we report that some differentially methylated probes can be detected in blood samples on average 3 years prior to the presence of clinical symptoms, which differentiate those with preclinical dementia compared to controls. More studies are needed to ascertain the true clinical utility of these DNAm signatures and the potential that they could be used in routine screening as an early biomarker of dementia. Future work is now also needed to determine whether these signatures are present even earlier in the disease process (eg, more than 3 years prior to symptom onset).

ACKNOWLEDGMENTS

The authors acknowledge the dedicated and skilled staff in Australia and the United States involved in the ASPREE cohort. The authors also are most grateful to the ASPREE participants, who so willingly volunteered for this study, and the general practitioners and staff of the medical clinics who cared for the participants. The work was supported by the National Institute on Aging and the National Cancer Institute at the National Institutes of Health (U01AG029824); the National Health and Medical Research Council (NHMRC) of Australia (334047 and 1127060); Monash University and the Victorian Cancer Agency. Joanne Ryan is funded by an NHMRC Dementia Research Leader Fellowship (APP1135727). Peter D. Fransquet is funded by an RTP stipend PhD scholarship, awarded by Monash University and the Australian Government.

CONFLICTS OF INTEREST

Anne Murray reports receiving consulting fees from Alkahest, Inc. and reports grants from the National Institute on Aging. Raj Shah reports grants for clinical research regarding dementia and Alzheimer's disease from National Institutes of Health, the Centers for Medicare and Medicaid Services, the Department of Defense, and the Illinois Department of Public Health; being a noncompensated board member of the Alzheimer's Association–Illinois Chapter; and being the site principal investigator or subinvestigator for clinical trials for which his institution (Rush University Medical Center) is compensated (Amylyx Pharmaceuticals, Inc., Eli Lilly & Co., Inc., Genentech, Inc., Merck & Co, Inc., Navidea Biopharmaceuticals, Novartis Pharmaceuticals, Inc., Roche Holdings AG, and Takeda Development Center Americas, Inc.). Paul Lacaze, Richard Saffery, James Phung, Emily Parker, and Robyn L. Woods report no disclosures relevant to the manuscript.

The funders had no role in the study design; collection, analysis, and interpretation of data; writing of the report; and decision to submit the article for publication.

AUTHOR CONTRIBUTIONS

Anne Murray, Robyn L. Woods, Raj Shah designed and conceptualized the ASPREE study and played a major role in the acquisition of data. Emily Parker and James Phung were responsible for the collection, storage, and retrieval of biological specimens. Joanne Ryan designed this study. Joanne Ryan and Paul Lacaze provided funding. Peter D. Fransquet undertook all EWAS data cleaning and analysis and wrote the manuscript, with assistance from Joanne Ryan. All authors provided intellectual input into the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Fransquet PD, Lacaze P, Saffery R, et al. Blood DNA methylation signatures to detect dementia prior to overt clinical symptoms. *Alzheimer's Dement*. 2020;12:e12056. https://doi.org/10.1002/dad2.12056